PERTURBATION OF MAMMALIAN CELL DIVISION

II. STUDIES ON THE ISOLATION AND CHARACTERIZATION OF HUMAN MINI SEGREGANT CELLS

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SUMMARY

A method is described for the isolation, according to size, of mini segregants produced by the abnormal cleavage of reversibly arrested mitotic HeLa cells. Many of these mini segregants contain small amounts of DNA, as judged by Feulgen staining and chromosome analysis. After fusion with mitotic HeLa cells, the interphase chromosomes of the mini segregants are seen as either monovalent or bivalent prematurely condensed chromosomes (PCC), some of which are damaged. A proportion of isolated mini segregants synthesize DNA, RNA and protein. Fusion of mini segregants with interphase HeLa cells gives rise to cells with 'hybrid' karyotypes.

INTRODUCTION

We have previously described a method for the production of HeLa cells which contain small amounts of DNA (Johnson, Mullinger & Skaer, 1975). These cells have been called 'mini segregants' and are the result of aberrant cytokinesis and chromosome segregation in mitotic cells. Details of some of the factors which influence the kinetics and characteristics of this process have been reported previously (Johnson et al. 1975), and further studies are in progress. Mini segregants are produced when mitotic cells are stored in the cold and then returned to normal growth conditions. After this treatment a proportion of the cells do not cleave normally, but produce a cluster of bud-like protuberances, resembling a bunch of grapes and referred to as 'BOGs'. DNA passes into many of the buds, which may subsequently detach from the parent BOG to form mini segregants. Cells remaining after the separation of the buds often contain reduced amounts of DNA and are also referred to as mini segregants. The resultant cell population is heterogeneous and consists of a mixture of mono- and multinucleate cells varying in size and morphology. These include mitotic and $G_1$ cells, BOGs and finally mini segregants, both with and without DNA.

Here we describe a method, based on sedimentation at unit gravity acceleration through a Ficoll density gradient, for the resolution of the heterogeneous cell mixture into a number of fractions each enriched for mini segregants of a given size range. These have been characterized in terms of DNA and chromosome content, capacity for macromolecular synthesis and ability to fuse with interphase cells.
MATERIALS AND METHODS

Production of mini segregants

HeLa cells with a modal chromosome number of 64 (Waldren & Johnson, 1974) were grown in the presence of 2.5 mM thymidine for 22 h and then arrested in mitosis by means of a nitrous oxide block at 5 atmospheres (5.066 x 10^6 N m^-2) (Rao, 1968). Routinely over 95% of the cells were held in mitosis and passed into interphase upon return to normal conditions of incubation. In order to produce mini segregants, the mitotic cells in Eagle's Minimal Essential Medium (Eagle, 1959) supplemented with 5% foetal bovine serum, pH 7.6, were cooled to 4 °C and stored thus for 9 h. The cells were then plated out into 150-mm plastic dishes (Falcon Plastics, Inc.) at 5 x 10^6 cells per dish and placed in a humidified CO₂ incubator at 37 °C. Abnormal cleavage began within 2 h, and at least 60% of the mitotic cells had formed BOGs after incubation for 4 h. These cultures were used for isolation of the mini segregants.

In some experiments mini segregants with labelled DNA were prepared. The cells were grown for one generation (21 h) in the presence of [³H]thymidine (0.2 μCi/ml) before the addition of the 2.5 mM thymidine block, and then in 1 μCi/ml [³H]thymidine after release from the thymidine block and before the beginning of the nitrous oxide pressure arrest.

Isolation of the mini segregants

Mini segregants were separated on the basis of cell diameter by sedimentation at 1 g through a 1–2% Ficoll density gradient. All operations were carried out at room temperature. The apparatus used (modified from Denman & Pelton, 1973) is shown in Fig. 1. The products of abnormal cleavage were gently pipetted to break up aggregates and to liberate as many buds as possible from the BOGs. Material derived from between 4 and 5 x 10^7 mitotic cells was resuspended in 30 ml of MEM and placed in the gradient apparatus. The Ficoll gradient was then introduced below the cells at a slow rate (approximately 4 ml/min) until the meniscus reached the side walls of the apparatus and the cells were confined to a narrow zone near the top of the gradient. The remainder of the gradient was then introduced at a faster rate (approximately 15 ml/min) until the meniscus reached the top of the cylindrical section. The cells were allowed to sediment through the gradient for 5 h, at which time 1 M sucrose was introduced from below, thus forcing the contents of the gradient out through the top exit tube (see legend, Fig. 1, for details); 25 fractions (50 ml each) were collected in this manner. After centrifugation, the supernatant was removed from each fraction and replaced with complete medium. The fractions from the gradient were numbered 1–25 and several fractions were pooled in groups for particular experiments (e.g. pooled fractions A, gradient fractions 1–8; pooled fractions B, gradient fractions 9–16; pooled fractions C, gradient fractions 17–24). There was some variation in the exact composition of the fractions prepared on different occasions.

Characterisation and fusion of the mini segregants

Cell and nuclear diameters were measured using a micrometer eye-piece and viable cell determinations made using trypan blue exclusion (0.4% trypan blue in phosphate-buffered saline).

The DNA content of individual cells was determined by Feulgen-DNA staining and scanning integrating microdensitometry. Cells were fixed in a large excess of neutral buffered formaldehyde solution for 24 h (Lillie, 1965), and then dropped on to glass slides and air dried. The slides were hydrolysed in 5 N HCl for 40 min at 20 °C, stained with Schiff's reagent, according to standard procedures (Pearse, 1968), and lightly counterstained with fast green. The density of the Feulgen staining was measured with a Vickers M85 Microdensitometer at 546 nm.

The nature of the chromosomes in the isolated mini segregants was studied by the technique of premature chromosome condensation (PCC) as described by Johnson & Rao (1970). Chromosome preparations were made according to standard techniques and stained with toluidine blue before examination.

Samples for autoradiography were extracted in 5% trichloroacetic acid (TCA) at 4 °C and
Fig. 1. Apparatus for separating mini segregant cells. The sedimentation chamber (A in diagram: 14.5 cm diameter, 17 cm height, approximately 1800 ml capacity) consists of a cylindrical plastic (Rohm & Haas Plexiglass) midpiece fitted into 2 Plexiglass blocks which form the lower and upper portions of the chamber. Rubber O-rings ensure water-tight connections, and the entire chamber is bolted together by 4 metal rods. Liquid enters the chamber from below through a perforated plastic disk covered with glass beads which reduce the turbulence and increase the resolution of the gradient. Reservoirs B and C are connected to the chamber by means of Teflon tubing via a 3-way valve. Screw clamps are located between all the compartments to control liquid flow. The contents of reservoirs C and D are mixed by means of magnetic stirrers.

The tubing connecting A with both B and C is first primed with medium (Eagle's MEM) and 50 ml are also introduced into A. Reservoirs C, D and E are filled with, respectively, 0.31% (150 ml), 1% (800 ml) and 2% (800 ml) Ficoll in MEM. The cell suspension containing the mini segregants is then added to B and allowed to flow slowly into A. Fresh medium is used to clear the connecting tube of cell suspension. The linear step gradient is formed by allowing the contents of reservoirs C, D and E to flow into the sedimentation chamber. The flow rate is adjusted by a screw clamp between A and C to approximately 4 ml/min for the first 150 ml and then to 15 ml/min for the rest. The cells are allowed to sediment for the desired time and then fractions are collected by replacing the contents of reservoirs C, D and E with 1 M sucrose which is allowed to flow into the chamber. The contents of the gradient are thus forced through the top exit and are collected in a series of 50-ml tubes.
coated with G5 emulsion (Ilford Ltd.) diluted 1:2 with 1% glycerol. Emulsion thickness was standardized by an automatic dipping machine. Slides were exposed for 3 weeks at 4 °C before development in D19 and subsequent staining in toluidine blue.

**Chemicals and radiochemicals**

[5-\(^{3}H\)]uridine, 25 Ci/mmol, L-[4,5-\(^{3}H\)]leucine, 46 Ci/mmol and [Me-\(^{3}H\)]thymidine, 17 Ci/mmol, were obtained from the Radiochemical Centre, Amersham. Other chemicals were obtained from Sigma Ltd.

**RESULTS**

**Isolation and characterization of the mini segregants**

Incubation of mitotic HeLa cells at 37 °C after a period of cold storage results in the abnormal cleavage of up to 60% of the population within 4 h. Careful collection and concentration of these cells provides an initial mixture which is heterogeneous, consisting of mono- and multinucleate cells of various sizes, including the mini segregant cells. Many of the smaller cells do not contain DNA. The initial mixture also includes a number of cells in which cleavage is incomplete (BOGs). We have used a gradient procedure similar to that described by Denman & Pelton (1973) to resolve the initial mixture of cells into a number of fractions, each enriched for mini segregants of a particular size range (see Fig. 1 for details). Each fraction was monitored and several fractions containing cells of approximately the same size were pooled to obtain sufficiently large samples. Photographs of the initial heterogeneous cell population and various fractions are shown in Figs. 6-9.

Both the initial mixture and the isolated fractions were tested for the exclusion of trypan blue. Approximately 95% of the cells in each sample excluded the dye and were therefore judged viable by this criterion. A considerable amount of cell debris accumulated during the period of incubation at 37 °C. This was easily distinguished from intact cells and was not included in the determinations of trypan blue exclusion. The debris tended to remain near the top of the gradient and was therefore primarily confined to the fractions containing the smallest mini segregants.

Cells in the initial mixture varied in size between about 1 and 30 \(\mu\)m (measured on living material). The pooled fractions were each enriched for cells in one of the following size ranges: below 6, 6 to 12, and 12 to 20 \(\mu\)m diameter (Fig. 2).

The distribution of DNA in cells of the initial mixture and in various pooled fractions was examined in fixed material stained by the Feulgen procedure. About 40% of the cells in the initial mixture had no Feulgen-positive staining and were apparently anucleate cell fragments (cytoplasts, Fig. 12). The remaining 60% of cells each contained one or more Feulgen-positive areas, but these showed considerable variation in size and appearance between different cells and even within one cell (Figs. 11–26). Two broad categories of Feulgen-positive bodies could be distinguished. The first type (which will be referred to as 'normal nuclei') resembled interphase nuclei since they contained areas of different staining intensity (Figs. 22–24), but they tended to be smaller than the nuclei of a random population of HeLa cells. (In formalin-fixed material 'normal nuclei' ranged in diameter between 1.5 and 9.5 \(\mu\)m, and the majority
were between 3 and 6 \( \mu \text{m} \), whereas the interphase nuclei of random HeLa cells fell within the range 5.5 to 10 \( \mu \text{m} \). The second type ('dense nuclei') appeared considerably condensed, stained uniformly at high intensity and were usually spherical in shape (Figs. 13–21); many fell within the same size range as the first type but others were below 1.5 \( \mu \text{m} \) in diameter. A small number of intermediate types were also found.

'Normal nuclei' and 'dense nuclei' were occasionally observed within the same cell (Figs. 25, 26) but more commonly, when a cell contained more than one stained body, they were all of the same type. The distribution of the different types of 'nuclei' in cells in the initial mixture and various fractions is shown in Table 1. The percentage of cells with any detectable Feulgen-positive area increased from 15% in pooled fractions...
Table 1. Classification of different cell types in the initial mixture and in isolated mini segregant fractions

<table>
<thead>
<tr>
<th>Sample</th>
<th>% of cells with Feulgen DNA stain</th>
<th>Cells with 'normal nuclei' only</th>
<th>Cells with 'dense nuclei' only</th>
<th>Cells with both 'normal' and 'dense nuclei'</th>
<th>Cells with mitotic chromosomes only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% of total population</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1     2    3     4     &gt;4</td>
<td>1     2    3     4     &gt;4</td>
<td>1     2    3     4     &gt;4</td>
<td></td>
</tr>
<tr>
<td>Initial mixture</td>
<td>58</td>
<td>39.6  15.9  8.4  3.0  3.0</td>
<td>10.8  2.7  0.0  2.7  5.5</td>
<td>2.0  6.4</td>
<td></td>
</tr>
<tr>
<td>Fractions 1-3 pooled</td>
<td>15</td>
<td>25.9  3.3  0.3  0.0  0.0</td>
<td>53.0  9.0  3.5  1.6  2.2</td>
<td>0.9  0.3</td>
<td></td>
</tr>
<tr>
<td>Fractions 4-5 pooled</td>
<td>29</td>
<td>33.0  4.3  0.7  0.2  0.0</td>
<td>37.0  10.7  4.5  3.8  2.4</td>
<td>2.4  1.0</td>
<td></td>
</tr>
<tr>
<td>Fractions 11-12 pooled</td>
<td>84</td>
<td>51.4  15.0  1.3  0.0  0.0</td>
<td>14.4  6.1  2.6  1.9  5.1</td>
<td>1.9  0.3</td>
<td></td>
</tr>
<tr>
<td>Fraction 14</td>
<td>78</td>
<td>39.9  12.7  3.7  0.6  0.0</td>
<td>20.8  7.2  2.9  2.3  8.4</td>
<td>0.9  0.6</td>
<td></td>
</tr>
<tr>
<td>Fraction 20</td>
<td>81</td>
<td>31.4  15.9  6.5  1.0  0.0</td>
<td>21.1  5.7  3.9  1.0  12.2</td>
<td>0.8  0.5</td>
<td></td>
</tr>
</tbody>
</table>

Counts of 300 cells were made for each sample. No distinction has been made between cells with nuclei of different sizes. Examples of the various morphological classes of mini segregants are shown in Figs. 11-26.
Perturbation of mammalian cell division. II

1-3 to 81% in fraction 20. Cells with two or more 'normal nuclei' were more common in fractions of higher number, and those with more than four 'normal nuclei' were observed only in the initial mixture, presumably because they came to lie in regions of the gradient below fraction 20. Cells with one 'dense nucleus' were particularly common in the fractions obtained from the top of the gradient.

![Diagram of Feulgen DNA values](image)

Fig. 3. Feulgen DNA values of cells in both the initial mixture which was layered on the gradient and in isolated fractions. Each sample included both mononucleate and multinucleate cells and also nuclei of both the 'normal' and 'dense' types (see text). 100 cells with Feulgen-positive staining were scored for each sample. The mean DNA Feulgen values with standard deviations are, in arbitrary units: initial mixture, 84.7 ± 48.9; fractions 1-3 pooled, 25.8 ± 27.2; fractions 4-5 pooled, 31.6 ± 32.1; fractions 11-12 pooled, 35.0 ± 16.1; fraction 14, 42.6 ± 14.4; and fraction 20, 85.7 ± 30.0. Arrows indicate the 2C and 4C Feulgen values for HeLa cells.

A quantitative estimate of the DNA content of the cells was obtained by microdensitometry of the Feulgen-stained material. As shown in Fig. 3, cells in the initial mixture showed Feulgen DNA values ranging from about 0.01 to 4C (compared with a random population of HeLa cells), and a relatively uniform distribution of values across this range. Values less than 0.01 C were outside the limits of detection under the conditions of measurement used. Cells in the isolated fractions had a narrower range of Feulgen values than those in the initial cell mixture, and the mean value increased from lower to higher fraction numbers. The wide range in staining intensities makes it difficult to give precise DNA values to the data given in Fig. 3, since the 'Feulgen artifact' is likely to be considerable, particularly for the 'dense nuclei' (Garcia, 1970).

The chromosome constitution of the isolated mini segregants was examined by the induction of premature chromosome condensation (Johnson & Rao, 1970). Prematurely
condensed chromosomes (PCC) are commonly induced when an interphase cell is fused with a cell in mitosis. The precise morphology of the PCC is a function of the position of the cell in interphase at the time of fusion. Thus, $G_1$ PCC consist of single chromatids, $G_2$ PCC of similar bivalent elements and $S$ PCC of a heterogeneous mixture of condensed and uncondensed regions.

**Table 2. Classification of PCC derived from isolated mini segregant fractions**

<table>
<thead>
<tr>
<th>Pooled fractions</th>
<th>$G_1$ PCC</th>
<th>$G_2$ PCC</th>
<th>Ambiguous PCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total = (normal) + (damaged)</td>
<td>Total = (normal) + (damaged)</td>
<td></td>
</tr>
<tr>
<td>A (gradient 1–8)</td>
<td>47 (25) (22)</td>
<td>39 (12) (27)</td>
<td>14</td>
</tr>
<tr>
<td>B (gradient 9–14)</td>
<td>38 (29.5) (8.5)</td>
<td>46 (21) (25)</td>
<td>16</td>
</tr>
<tr>
<td>C (gradient 17–24)</td>
<td>77.5 (64) (13.5)</td>
<td>19.5 (5) (14.5)</td>
<td>3</td>
</tr>
</tbody>
</table>

At least 200 chromosome spreads containing PCC were scored for each sample. Examples of the various morphological categories are shown in Figs. 27–32.

In order to obtain a sufficient number of cells for the analysis of PCC, 50-ml fractions taken from the gradient were pooled to form 3 larger fractions. Aliquots of the pooled fractions were then fused with mitotic cells to induce PCC. Control mitotic × mitotic fusions were carried out in order to characterize the contaminating PCC arising from interphase cells present in the mitotic population. In addition, fusion between mini segregants pre-labelled with [H]thymidine and unlabelled mitotic cells clearly established the origin of the PCC (Fig. 32) and showed that it was not in general necessary to use pre-labelled material in order to recognize the mini segregant PCC.

Chromosome spreads most commonly contained single clusters of PCC which were either $G_1$, bivalent or damaged. The bivalent PCC were often attenuated, suggesting that they had undergone a considerable degree of decondensation from their initial mitotic condition (Fig. 30). The $G_1$ PCC also varied in their degree of attenuation (Figs. 27, 28). A number of PCC displayed various degrees of damage, frequently in the form of localized breaks, despiralization or fragmentation (Fig. 31). The frequency with which these different types of PCC occurred in the different fractions is shown in Table 2. Although each fraction contained both $G_1$ and bivalent PCC, the ratio of $G_1$ to bivalent tended to increase with increase in mini segregant size. Damaged PCC were more common in the smaller fractions. Occasionally chromosome spreads were seen with more than one cluster of PCC. Such spreads may have been the result either of fusion involving several mini segregants or fusion with a single mini segregant.
Fig. 4. Histograms showing the distribution of mini segregant cells containing different numbers of PCC in various pooled fractions: A, from gradient fractions 1–8; B, from gradient fractions 9–16; and C, from gradient fractions 17–24. These data are based on the same isolation as that in Fig. 2. Each pooled fraction was fused with mitotic HeLa cells to induce PCC. Chromosome preparations were made and the number of prematurely condensed chromosomes in at least 200 PCC clusters was counted for each fusion sample. The mean chromosome numbers with standard deviations in the pooled fractions are: A, 17.3 ± 11.7; B, 26.5 ± 12.9; and C, 35.7 ± 12.2. Few spreads containing PCC were observed in control mitotic × mitotic fusions. These were invariably of standard G2 morphology and displayed a tight distribution around 64, the modal chromosome number for this strain of HeLa cell.
containing more than one nucleus. The same morphology of PCC was always seen within a given cluster, although all the clusters in one chromosome spread were not necessarily of the same type (Fig. 30). For purposes of counting and classifying the mini segregant chromosomes, only those chromosome spreads containing one cluster of PCC were scored. This will have resulted in an underestimate of the chromosome constitution of the mini segregant population, although the low frequency of spreads with more than one cluster is probably not great enough to influence the results significantly.

The distribution of cells containing different numbers of chromosomes in 3 pooled fractions is shown in Fig. 4 and should be compared with the distribution of cell sizes presented in Fig. 2. A good correlation was found between chromosome number and cell diameter, the smaller cells generally containing fewer chromosomes than the larger. The mean chromosome numbers (with standard deviations) in the various fractions were: A (fractions 1–8), 17.3 ± 11.7; B (fractions 9–16), 26.0 ± 12.9; and C (fractions 17–24), 35.7 ± 12.2. Cells from pooled fraction A generally contained a greatly reduced chromosome complement. PCC consisting of one discrete chromosome were seldom observed, although the Feulgen values suggest that many of the smallest mini segregants contained only a single chromosome or chromosome fragment. In the control mitotic × mitotic fusion, few PCC preparations were observed. These were of G2 morphology and displayed a tight distribution of chromosome number around 64. They probably resulted from a low level of G2 contamination in the mitotic population.

**Synthesis of macromolecules by isolated mini segregants**

The ability of isolated mini segregants to synthesize DNA, RNA and protein was studied in the following manner. Fractions A and B (average chromosome numbers of 17.3 and 26.0 respectively) were each incubated in plastic dishes in medium supplemented with either 1 μCi/ml [3H]thymidine (24 h), 3 μCi/ml [3H]uridine (3 h) or [3H]leucine (5 μCi/ml). Cells were deposited on slides with a cytocentrifuge and processed for autoradiography, as described in Materials and methods. The numbers in parentheses denote the total number of cells scored for each sample.

<table>
<thead>
<tr>
<th>Synthesis</th>
<th>Length of exposure to radioactive precursor, h</th>
<th>Pooled fraction</th>
<th>% of nucleated cells labelled</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>24</td>
<td>A</td>
<td>6 (236)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>34 (202)</td>
</tr>
<tr>
<td>RNA</td>
<td>3</td>
<td>A</td>
<td>14 (176)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>52 (341)</td>
</tr>
<tr>
<td>Protein</td>
<td>3</td>
<td>A</td>
<td>47 (321)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>57 (251)</td>
</tr>
</tbody>
</table>

Pooled fractions A and B (mean chromosome numbers 17 and 26 and pooled gradient fractions 1–8 and 9–16, respectively) were incubated in the presence of [3H]thymidine (5 μCi/ml), [3H]uridine (5 μCi/ml) or [3H]leucine (5 μCi/ml). Cells were deposited on slides with a cytocentrifuge and processed for autoradiography, as described in Materials and methods. The numbers in parentheses denote the total number of cells scored for each sample.
3 μCi/ml [3H]leucine (3 h). Samples were removed from the dishes after incubation and processed for autoradiography. Since a number of the mini segregants adhered to the plastic surface, samples were trypsinized and cytocentrifuge preparations made of the pooled floating and attached cells.

The results of these experiments (Figs. 33, 34 and Table 3) showed that a proportion of nucleated segregants in each of the pooled fractions retained the ability to synthesize DNA, RNA and protein. There was no evident synthesis in the cytoplasts. Considerably fewer nucleated cells in the smaller fraction (A, gradient fractions 1–8) incorporated either thymidine or uridine as compared with the larger fraction (B, gradient fractions 9–16). There was less discrepancy between the two fractions in their ability to incorporate leucine. A more detailed analysis of the incorporation of uridine and leucine by isolated mini segregants is shown in Fig. 5, in which the average number of silver grains is plotted as a function of the nuclear diameter. There is an approximately linear relationship between the amount of precursor incorporated per cell and nuclear diameter. Amongst those cells which synthesized RNA there was variation in the localization of the radioactivity within the cells. In some, the radioactivity was largely confined to the nucleus, while in others both nucleus and cytoplasm were equally labelled.
Integration of mini segregant chromosomes into hybrid cells

One potential use of mini segregants depends on the integration of their chromosomes into the genome of hybrid cells produced by cell fusion. We have investigated this problem by producing mini segregants with labelled DNA, and following the fate of the labelled mini segregant chromosomes during the first and second cell cycles after fusion with unlabelled random interphase HeLa cells. Several hours after fusion numerous heterokaryons were observed which contained one or more highly labelled micronuclei (Fig. 35). Chromosome preparations were made from cells collected during the first and second mitosis after fusion and a proportion contained labelled chromosomes or micronuclei of mini segregant origin (Figs. 36, 37). A number of the chromosomes were heavily labelled, whereas others showed local concentrations of radioactivity (Fig. 36). These observations show that chromosomes (or chromosome fragments) derived from mini segregants are incorporated into hybrid nuclei. Occasionally, in the first mitotic figures after fusion, areas of radioactivity were found which were not associated with distinct chromosomes (Fig. 38). These may have arisen from the highly damaged mini segregant chromosomes observed by the analysis of PCC (Fig. 31).

DISCUSSION

We have previously described a method for promoting aberrant chromosome segregation and cytokinesis in mitotic HeLa cells (Johnson et al. 1975). This procedure involves storage of mitotic cells in the cold (4 °C) before continued incubation at 37 °C. A number of factors, including the duration of cold storage, the pH of the medium and the presence of serum and thiol compounds, were found to influence the pattern and kinetics of abnormal cleavage. The present paper is concerned with the isolation and characterization of the cells resulting from this process. We have chosen to induce abnormal cleavage under relatively gentle conditions, namely in complete MEM at pH 7.6 after a 9-h period of cold storage, in order to increase the probability of cell survival.

It is possible to resolve the initial population of cells into several fractions, each enriched for a particular size range. The technique involves sedimentation through a 1–2% Ficoll density gradient. Cells are allowed to fall through the gradient under unit gravity acceleration at a rate predominantly determined by cell diameter and shape. Similar procedures based on sedimentation at unit gravity acceleration are capable of resolving rather small differences in cell diameter and have been utilized successfully to separate cells derived from bone marrow (Peterson & Evans, 1967), erythrocytes (Miller & Phillips, 1969), mouse fibroblasts (Shall & McLelland, 1971) and cell nuclei (McBride & Peterson, 1970).

The initial population which is placed on the gradient consists primarily of cells with single or multiple DNA-containing areas and anucleate cytoplasmic bodies (cytoplasts). Under the conditions of sedimentation employed, most of the cytoplasts come to lie in fractions collected from the top of the gradient, where they are mixed with mini segregants of small size which contain DNA.
The fractions isolated from the gradient have been characterized both in terms of DNA content and chromosome number. Feulgen-DNA values increase with increasing cell size and nuclear diameter. There is a good correlation between the DNA content and chromosome number in cells isolated from different regions of the gradient. Since many of the mini segregants would probably never divide, the chromosome number has been determined by counting the prematurely condensed chromosomes (PCC) produced by fusion with mitotic cells (Johnson & Rao, 1970). Each of the fractions described here contains segregants with a reduced complement of chromosomes. Mini segregants in the smallest fraction contain an average chromosome number of 17.3, substantially below the haploid human value. Very few mini segregant PCC consist of a single discrete chromosome, although the Feulgen analysis revealed a large proportion of cells with small amounts of DNA. The disparity in these data may result from an inability of the smallest DNA-containing cells to fuse, or more likely, from the overlooking of small and probably fragmented PCC among the chromosome spreads. The probability of scoring multiple fusions was reduced in these experiments by adjusting the ratio of mitotic to mini segregant cells in the fusion mixture to approximately 4 to 1. Moreover, any such multiple fusions as occurred would be excluded from the analysis, since the resultant PCC tend to lie in separate areas of the chromosome spread and may even display different chromosomal morphologies (Fig. 30).

The analysis of mini segregant PCC reveals that in each fraction there are substantial numbers of cells containing either $G_1$ or bivalent chromosomes. Mixtures of $G_1$ and bivalent PCC in the same group of PCC have not been observed. There is considerable damage in many of the PCC, taking the form of either fragmentation or lightly staining areas. Damage of this nature is most abundant in cells from the smallest fraction, although segregants from all regions of the gradient which contain bivalent chromosomes show a greater frequency of damage than their $G_1$ counterparts. The reason for the greater proportion of $G_1$ PCC in the largest fraction is not clear, but it is possible that the larger the segregant, the more normal was both the cleavage of its parent cell and the segregation of sister chromatids prior to division.

We suggest that there is a relationship both between the ‘dense nuclei’ and bivalent PCC, and between the ‘normal nuclei’ and $G_1$ PCC. The enrichment of the smaller fractions in both bivalent PCC and ‘dense nuclei’ lends support to this idea. The production of the two forms of ‘nuclei’ during the abnormal cleavage, resulting either in mini segregants containing exclusively one or the other type or, more rarely, multinucleate cells in which both types are present, cannot yet be adequately explained. The fact that chromosomal damage is more common in bivalent than $G_1$ PCC in these segregant cells supports the idea that bivalent prematurely condensed chromosomes and ‘dense nuclei’ result from more severely perturbed divisions. The absence of a complete spindle structure may be an important factor both in abnormal separation of sister chromatids and in the production of chromosome damage.

The isolated mini segregants have been tested for their ability to synthesize DNA, RNA and protein. In these experiments the analysis of uptake of radioactive precursors into TCA-insoluble macromolecules has been assessed for the total population of
nucleated cells in both the pooled fractions used. Many of these cells did not attach to the plastic dishes and it is possible that they died during the period of incubation. Further studies are in progress to clarify this point. A few (6%) of the nucleated cells in the smaller pooled fraction (average chromosome number = 17) were able to initiate DNA synthesis during a 24-h period following isolation. A considerably larger proportion (34%) of the larger fraction (average chromosome number = 26) began DNA synthesis under the same conditions. Since these cells might correspond to the larger end of the size range in each fraction it is important to determine both the chromosomal constitution of those mini segregants capable of DNA synthesis and also the extent to which replication proceeds in cells with different karyotypes. Since many of the mini segregant PCC are bivalent in type, and often substantially decondensed, it is possible that these elements are capable of sustaining DNA synthesis. In addition, those cells which emerge from cytokinesis with bivalent chromosomes can be followed in the post-mitotic cell in order to determine whether there is a progressive chromosome decondensation typical of normal $G_1$ (Schor, Johnson & Waldren, 1975), or a progressive condensation typical of $G_2$ (Sperling & Rao, 1974).

Mini segregants are also found to be capable of synthesizing RNA and protein. A good correlation is found between the amount of precursor incorporated and the nuclear diameter. Many of the mini segregants show differences in [3H]uridine labelling patterns after administration of a 3-h pulse. In some cells most of the label is nucleus-associated, while in others the cytoplasm also is heavily labelled.

The ability of mini segregants to fuse with either mitotic or interphase cells offers a powerful means of introducing small quantities of DNA which become part of the genetic constitution of the hybrid cell. Mitotic figures at the first and second divisions after fusion show the inclusion of chromosomes from the mini segregant cells.

There are now two methods for the production of cells containing small quantities of DNA. Stubblefield (1964) showed that Chinese hamster cells could be induced to form karyomeres in the presence of colcemide. Recently Ege & Ringertz (1974) demonstrated that rodent cells induced to form karyomeres in the presence of colcemide can be enucleated by treatment with cytochalasin B to produce micro cells which can be fused with normal mitotic cells (Ege, Krondahl & Ringertz, 1974; Veomett, Prescott, Shay & Porter, 1974). The present study is based on mini segregants produced by aberrant cytokinesis and chromosome segregation in mitotic HeLa cells, an aneuploid line of human origin. We find that primary human diploid cells can also be induced to form mini segregants (in preparation). The combination of cell fusion and mini segregant or microcell formation now offers new opportunities in the study of somatic cell genetics.

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Figs. 6–9. Photomicrographs of the initial mixture which was layered on the gradient (Fig. 6), and of isolated gradient fractions 1–2 (Fig. 7), fractions 9–10 (Fig. 8) and fractions 19–20 (Fig. 9). × 330.
Fig. 10. Photomicrograph of HeLa cells arrested in mitosis. × 330.
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Fig. 11. Photomicrograph of pooled fractions 4 and 5 stained by the Feulgen DNA technique and lightly counterstained with fast green. × 250.

Figs. 12-26. Photomicrographs of different types of cells observed in the initial mixture which was layered on the gradient, stained by the Feulgen DNA technique and lightly counterstained with fast green. × 1400.

Fig. 12. Cytoplasts with no Feulgen-positive area.

Fig. 13. Small cell with 1 small 'dense nucleus'.

Fig. 14. Larger cell with 1 small 'dense nucleus'.

Fig. 15. Cell with 1 'dense nucleus'.

Fig. 16. Cell with 2 'dense nuclei'.

Fig. 17. Cell with 2 'dense nuclei', one small and one large.

Figs. 18-20. Cells with many 'dense nuclei'.

Fig. 21. BOG with 2 'dense nuclei'.

Fig. 22. Cell with 1 'normal nucleus'.

Fig. 23. Cell with 3 'normal nuclei'.

Fig. 24. Cell with 4 'normal nuclei'.

Fig. 25. Cell with several 'normal nuclei' and several 'dense nuclei'.

Fig. 26. Cell with 1 'normal nucleus' and 2 'dense nuclei'.
Figs. 27–32. Prematurely condensed chromosomes (PCC) induced from mini segregant cells after fusion with mitotic HeLa cells. × 2000.

Fig. 27. Mini segregant PCC containing about 30 condensed G1 elements.

Fig. 28. Mini segregant PCC containing about 10 attenuated G1 elements.

Fig. 29. Chromosome spread containing 7 condensed PCC that are probably bivalent.

Fig. 30. Chromosome spread containing 3 extended and damaged bivalent PCC and a separate group of damaged, condensed G1 PCC (arrow). This cell may be the consequence of either multiple fusion or fusion with a multinucleate mini segregant.

Fig. 31. Mini segregant PCC showing highly fragmented elements.

Fig. 32. Autoradiograph of G1 PCC from a mini segregant derived from a mitotic cell which had been pre-labelled with [3H]thymidine.
Figs. 33, 34. Incorporation of $^3$H-precursors into TCA-insoluble products in mini segregant cells from pooled fraction B. Cytocentrifuge preparations. $\times 1200$.

Fig. 33. Incorporation of $[^3H]$uridine after a 3-h incubation period.

Fig. 34. Incorporation of $[^3H]$leucine after a 3-h incubation period.

Figs. 35–38. Products of fusion between mini segregant cells labelled with $[^3H]$thymidine and random HeLa cells, fixed during the first and second cycles after fusion. $\times 1200$.

Fig. 35. Cytocentrifuge preparation of multinucleate cell, from the first interphase after fusion, containing one mini segregant nucleus (identified by silver grains) and 7 HeLa nuclei.

Fig. 36. Hybrid metaphase chromosome spread, from the second mitosis after fusion, showing heavily labelled chromosomes (h) of mini segregant origin, and also a more lightly labelled chromosome (l) presumably arising from chromosome rearrangements within the fused cell.

Fig. 37. Metaphase chromosome spread associated with 2 small pre-labelled mini segregant nuclei.

Fig. 38. Metaphase chromosome spread with adjacent region of radioactivity which is presumably associated with highly decondensed mini segregant chromatin.
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