A CELL-CYCLE-PHASE-SPECIFIC MUTANT OF AMOEBA

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SUMMARY

The treatment of Amoeba indica with ethylmethanesulphonate (EMS) at early S, late S and late G2 phases of the cell cycle leads to the production of mini amoeba cells in the G2 period. Among them, only a few of the mini cells that originated from EMS treatment at early S phase have been found to be viable and to give rise to stable clones. These mini amoebae show stable and altered characteristic features in cell size, structure, membrane properties, cell-cycle timing and the patterns of macromolecular syntheses as compared to the parental cells. It is suggested that the mini amoeba cell is a size mutant that has a cell-cycle-phase-specific origin. The finding is discussed in relation to preferential mutagenic action involving the functional state of DNA leading to the production of viable mutant amoebae.

INTRODUCTION

Ethylmethanesulphonate (EMS) has been proved to be an effective mutagen for prokaryotes and several eukaryotes (Schalet, 1978). The highly effective mutagenicity of EMS has been shown in Escherichia coli (Loveless & Howarth, 1959), Drosophila (Alderson, 1964), Neurospora (Malling & De Serres, 1968) etc. The use of chemical mutagens in amoeba has been undertaken only during the last few years. Mutations have been induced in Amoeba proteus by treatment with N-methyl-N-nitrosourethane (MNU) (Ord, 1965, 1970). Three mutants have been obtained following treatment of amoebae with MNU at mid-G2, late G2 and S phases. In the giant amoeba, Chaos chaos, X-irradiation during division resulted in the production of stable clone of smaller amoebae (Schaeffer, 1946). However, no evidence is available to confirm that this cell type is a mutant. Some temperatursensitive and antibiotic-resistant mutant amoebae of Physarum have also been isolated recently following treatment with N-methyl-N'-nitro-N-nitrosoguanidine and EMS (Burland & Hangli, 1982; Evans, 1982).

The present investigation is concerned with the action of EMS at various phases of the cell cycle of a unicellular, mononucleate, eukaryotic cell, Amoeba indica. The amoebae were exposed to the mutagen for a brief period at every hour throughout the cell cycle. Our study shows that treatment of A. indica with EMS at a specific phase of the cell cycle leads to the production of 'mini' cells that are viable and produce a stable clone showing several altered features. We describe here the origin, isolation and characteristics of these mini amoebae, which have been cultured in our laboratory

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for more than 3 years undergoing over 800 generations and still maintaining the altered characteristic features.

MATERIALS AND METHODS

Cells and culture methods

A. indica (Rao, 1971) was used as an experimental organism for the present investigation. Amoebae were cultured at pH 6-8 and according to the method of Chatterjee & Rao (1974). All the cultures were maintained at 21 ± 1 deg. A. indica had no detectable G₁ phase and had a generation time of 24 ± 2 h. Its S phase covered 3 ± 1 h and the rest of the period had been considered to be G₂ phase (Rao & Chatterjee, 1974). However, under our laboratory conditions the generation time of A. indica was found to differ from that reported by those authors. The S phase covered 7 h following mitosis and the duration of G₂ phase was 64 ± 6 h. The mitotic phase took approx. 35–40 min. The entire investigation on the mutant amoebae was carried out about 2 years after their origin, when they had undergone over 600 generations.

The mini amoebae, obtained following EMS treatment, were cultured by adopting the capillary cloning method (Ord, 1979a) to produce a clone. The method was used to avoid contamination by other amoebae in the mini cell culture and to observe the progeny obtained from a single mini cell. Modified Chalkley’s medium at pH 6-8 (Ord, 1970) was mainly used both for capillary cloning and routine culture of the mini amoebae. Cultures were maintained at 21 ± 1 deg. C. Chitomonas was used as food for the mutant cells.

EMS treatment

For EMS (Sigma Chemical Co., St Louis, U.S.A.) treatment, the desired volume was added to 0-5 ml of Analar ethanol, and the mixture was then diluted with sterile amoeba medium to give a concentration of 0-5 % (pH adjusted to 6-8). The mitotic cells were picked up from the culture and different groups of daughter cells were exposed to 0-5 % EMS solution for 15 min at 1-h intervals throughout the cell cycle including the mitotic phase. The treated cells were washed thoroughly with amoeba medium and kept either singly or in groups of 20 in Syracuse watch-glasses. Appropriate controls (untreated amoebae of similar age) were kept in each case. Both treated and the control amoebae were usually fed 1 h after exposure to the mutagen, with a light suspension of Tetrahymena.

Light microscopy

Photographs of living cells were taken with a Carl Zeiss ‘Tessovar’ Photomacrographic Zoom system. Cell size and nuclear diameter for both living and fixed cells were measured by placing an ocular micrometer in the eyepiece of a Carl Zeiss ‘Amplival’ microscope. For squash preparations, amoebae were flattened with a coverglass containing 45 % (v/v) glacial acetic acid and then the slide was dipped in liquid nitrogen and the coverglass was flipped off. Cells were postfixed in acetic acid/ethanol (1:3, v/v), passed through a graded series of ethanol and finally air-dried. Preparations were stained with Giemsa.

Scanning electron microscopy

Amoebae were placed on gelatinized coverslips and fixed for 1 h in Karnovsky’s (1965) fixative, containing 5 % glutaraldehyde and 4 % formaldehyde in 0-1 M-cacodylate buffer (pH 7-3), rinsed in buffer and postfixed for 1 h in 1 % OsO₄ in 0-1 M-cacodylate buffer (pH 7-3). Cells were then washed in double-distilled water, dehydrated in a graded series of ethanol, and acetone and dried in a Sorval critical-point drier with liquid CO₂. The specimens were coated with gold in a vacuum and examined in a Cambridge Stereoscan, model S4-10 operated at 30 kV.

Transmission electron microscopy

The cells were fixed for 1 h in Karnovsky’s (1965) fixative and rinsed in distilled water. They were then embedded in a small agar cube (Flickinger, 1969). The samples were then postfixed in 1 %
OsO$_4$ in 0.1 M cacodylate buffer (pH 7.3), dehydrated in a graded series of ethanol followed by propylene oxide, and embedded in Araldite. Thin sections (0.5 µm) were cut and mounted on copper grids, and stained with uranyl acetate and lead citrate (Reynolds, 1963). The preparations were examined in a Siemens 1A electron microscope operated at 80 kV.

**Labelling of cells and autoradiography**

For DNA and protein syntheses, [3H] thymidine (sp. act. 17.8 Ci/mmol, Bhabha Atomic Research Centre (BARC), India) and [3H] leucine (sp. act. 3.3 Ci/mmol, BARC, India), respectively, were used. The division spheres were collected from the mass cultures and kept fed till they had reached the appropriate age. Cells were labelled for 30 min or 1 h through each point. The labelled pools were chased with an excess of unlabelled thymidine (3 × 10$^{-5}$ M, Sigma Chemical Co., U.S.A.) for 1 h or cold leucine (2 × 10$^{-3}$ M, Sigma Chemical Co., U.S.A.) for 15 min. The labelled cells were squashed and covered with Kodak AR 10 stripping films. After a suitable period of exposure the labelled preparations were developed, fixed and stained with Giemsa. The total number of silver grains over the whole nucleus were counted to determine thymidine labelling, using an eyepiece graticule in the ocular. For leucine labelling, an area of 2700 µm$^2$ was counted from three randomly selected cytoplasmic regions in each cell. Appropriate background corrections were made. The presence of at least 20 grains over the nucleus, and above the background level, was taken as an indication that the cell was in S phase.

**Enzyme extraction**

The specificity of [3H] thymidine incorporation was checked by DNase digestion. DNase solution at a concentration of 0.3 mg/ml (pH 7.0) was prepared and the digestion was carried out for 6 h at 37°C. Following enzymic digestion the preparations were washed in double-distilled water, air-dried and processed for autoradiography.

**RESULTS**

The exposure of early S and late G$_2$ phase amoebae to EMS was lethal to approximately 30% of the cells. The late S phase cells, however, appeared to be less affected by EMS treatment, as only approximately 15% cell mortality was recorded.

**Cell-cycle-phase-specific origin of viable and lethal mini amoebae**

It was noticed that when the early S phase (approx. 1 h after division) cells were treated with EMS some mini cells appeared in the treated population at around 36 ± 3 h after the treatment. Some mini cells were also found to originate around 69 ± 2 h and 72 ± 3 h of the cell cycle when the cells were treated with EMS at late S (approx. 51 h after mitosis) and late G$_2$ (approx. 69 h after division) phases, respectively (Fig. 1). However, the mini cells that originated during these two phases of the cell cycle were found to be non-viable, as they died within 15–20 days after their origin.

More than 1400 early S phase cells, obtained from several sets of experiments, were treated with EMS and the results of these experiments were grouped together. Immediately following treatment the early S phase cells were separated and kept singly for observation at 1-h intervals up to 72 h at least. The same procedure was also followed for late S phase (800 cells) and late G$_2$ phase (750 cells) treated cells. The mini cells that formed were isolated from their parental cells for further observation.
Fig. 1. A time model showing the appearance of mini amoebae after treating *A. indica* cells with EMS. The black arrow at the top indicates the point of exposure to mutagen that leads to the appearance of viable mini cells (black arrow at the bottom). The arrowheads indicate the points of treatment with mutagen that lead to the production of non-viable mini cells. Numbers within the circle denote hours. M, mitotic phase; S, S phase; EG2, early G2 phase; MG2, mid G2 phase; LG2, late G2 phase.

Fig. 2. Living cells showing the difference in size between *A. proteus* (left), a well-known species shown as a reference; a mini mutant of *A. indica* (middle) and the control *A. indica* (right). ×64.

Fig. 3. Difference in size between *A. proteus* (p), mini mutant (m) and *A. indica* (i). Cells were squashed and stained with Giemsa. ×93.

Fig. 4. Squashed cells showing ingestion of only one *Tetrahymena* (t) by each mini amoeba mutant. ×93.

Fig. 5. A squashed mini amoeba showing the formation of more than 20 phagosomes (p) after feeding with *Chilomonas*. ×400.
Cell cycle mutant of amoeba

Figs 2–5
Table 1. The cell size and nuclear diameter of A. indica and mini amoeba

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Average cell size (μm ± S.D.)</th>
<th>Average nuclear diameter (μm ± S.D.)</th>
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<tbody>
<tr>
<td>A. indica</td>
<td>397 ± 11</td>
<td>33 ± 2.5</td>
</tr>
<tr>
<td>Mini amoeba</td>
<td>224 ± 7</td>
<td>22 ± 1.8</td>
</tr>
</tbody>
</table>

The measurements were obtained from more than 100 living cells of mixed ages. ± S.D., standard deviation.

The mini cells that were found capable of producing phenotypically similar mini cells only were then cloned. Approximately 250 mini cells were collected from the culture of treated early S phase cells. The majority of these cells reverted back to the 'apparently normal' form about 2 months after their formation. Quite a few mini cells were also found to be non-viable within 5 months of their formation. Only 22 viable mini cells that produced stable clones could be isolated and they were cultured separately. The average size of mini cells was found to be smaller than control A. indica cells. Cell size was measured in living cells (Fig. 2) as well as in flattened and fixed cell (Fig. 3) preparations (Table 1).

Some characteristic features of mini amoebae

Membrane properties. The cell membrane of the mini amoebae was found to have developed a greater adhesiveness to the substratum as compared to control A. indica cells. This increased adherence was evident while picking up the cells from the culture dish with a braking pipette. The majority of the mini amoebae had pseudopodia with more or less pointed tips whereas in the parental cells the pseudopodial tips were hemispherical.

The cloned mini mutant amoebae were supplied with Tetrahymena for food. It was noticed that the mini cells could capture only one Tetrahymena at a time, although the food was available to them ad lib (Fig. 4). Moreover, the mini cells died after being fed with Tetrahymena for more than a day or two. It was noticed that the mini cells could capture more than 20 Chilomonas at a time (Fig. 5) without any deleterious effect. Therefore, Chilomonas feeding was adopted for raising the mini cell culture. The control A. indica, however, could feed equally well on both Tetrahymena and Chilomonas.

Fig. 6. Scanning electron micrograph of a portion of control A. indica cell surface with many protuberances (p). ×5250. Inset: a magnified view of a bleb. ×5400.

Fig. 7. Scanning electron micrograph of a portion of mini mutant cell surface with blebs (b), mini beads (mb) and pits (p). ×5250. Inset: a magnified view of a bleb with conical projections. ×5400.

Fig. 8. Part of a control amoeba showing many and varied types of crystals. Squash preparation, stained with Giemsa. ×640.

Fig. 9. Part of a mini amoeba mutant showing only a few spherical crystals. Squashed cell, stained with Giemsa. ×640.
Cell cycle mutant of amoeba

Figs 6–9
Comparative scanning electron microscopic analysis of the cell surface of control and mini mutant amoebae revealed several distinctive differences between them. The mini amoebae had innumerable pits on their cell surface, which were either absent or rarely visible in the control A. indica cells. The mutant cells had a large number of mini beads on their cell surfaces instead of the innumerable protuberances that were encountered in the parental A. indica cells (Figs 6, 7). While both types of cells had blebs on their cell surface, the mini cell blebs were found to contain conical projections on their surface that were absent in the control A. indica cells (Figs 6, 7).

Nuclear and cytoplasmic structures. The nuclear diameter of the mini amoebae was found to be quite small when compared with control A. indica. Measurements of random samples of nuclei from mini cells and A. indica made at all the cell cycle stages are given in Table 1. Cytoplasmic crystals were very rare in the mutant cells and had rounded contours. In contrast, abundant crystals having two to three types of configurations were found to occur in control A. indica cells (Figs 8, 9). The cytoplasm of the mini mutant cells also had a large number of vacuoles as compared to the control amoebae.

A great deal of difference was found between the nuclei of mutant amoebae and their parental cells. The mini amoeba nucleus was found to be surrounded by a layer of microfibrils, which was absent in the control A. indica cells. The nucleoli of the mutant cells were very dense and granulated and often contained intranucleolar fibrils. In contrast, the nucleoli of control cells were diffuse and in some cases contained dense nucleolar inclusions, but no intranucleolar fibrils could be detected in them. Abundant electron-dense particles were noted in the mini mutant nucleus, but were almost totally absent in the parental cells (Figs 10, 11). The chromatin of the mutant amoebae also appeared to be fairly dense as compared to the control cells, which were more diffuse (Figs 10, 11).

There was a remarkable structural difference between the endoplasmic reticulum of control A. indica cells and mutant amoebae. The configuration of the granular endoplasmic reticulum was found to be vesicular in mutant cells, whereas it appeared in the form of cisternae in control amoebae (Figs 12, 13).

Cell cycle timing and patterns of macromolecular syntheses. The generation time of the mini amoebae was found to vary considerably for several generations after their formation. However, when cell cycle studies were undertaken on these mini cells in a more systematic manner, approximately 2 years after their formation, they showed much shorter and more or less consistent cell cycle timing. The entire cell cycle of the

Fig. 10. Electron micrograph of a view of a portion of a control A. indica nucleus. nu, nucleoli; dr, dense nucleolar inclusions. ×18000. Inset: ch, chromatin. ×5000.

Fig. 11. Electron micrograph of a view of a portion of a mutant amoeba nucleus. mf, microfibrils; nu, nucleoli; if, intranucleolar fibrils; ep, electron-dense particles. ×18000. Inset: ch, chromatin. ×6050.

Fig. 12. Electron micrograph of rough endoplasmic reticulum (rer) of control A. indica. G, golgi body. ×32000.

Fig. 13. Electron micrograph of rough endoplasmic reticulum (rer) of mutant amoeba showing vesicular nature. ×32000.
Cell cycle mutant of amoeba

Figs 10-13
Table 2. Duration of different phases of the cell cycle of A. indica and mini mutants

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Phases of the cell cycle (h)</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td>A. indica</td>
<td>0.55–0.6</td>
<td>7</td>
</tr>
<tr>
<td>Mini amoeba</td>
<td>0.40–0.45</td>
<td>5</td>
</tr>
</tbody>
</table>

G.T., generation time; ± s.d., standard deviation.

The mini amoeba mutant was found to be completed within 20 ± 2 h compared to 72 ± 6 h for the parental cells. The mitotic phase of the mini amoebae was between 20 and 25 min. The S phase took 5 h while the G2 phase took 15 ± 2 h (Table 2). It was noted that there was no discernible difference in the duration of different phases of the cell cycle when the amoebae were cultured in either Prescott’s or modified Chalkley’s medium.

Autoradiographic analysis of the DNA synthesis period in the mutant amoebae showed several other differences from parental cells. Apart from the shortening of S phase, the peak of incorporation of [3H]thymidine occurred at around 3 h following...
mitosis in the mutant amoebae, as compared to the peak at 4 h in control cells. Another interesting finding was the very low incorporation of labelled exogenous thymidine into the mutant cells during the first half-hour following mitosis. In the corresponding period in the control amoebae more than 40% of the incorporation occurred, as compared to the peak of incorporation at 4 h (Fig. 14).

A comparative analysis of [³H]leucine incorporation throughout the cell cycle also demonstrated several notable variations in the protein synthetic pattern. The amount of labelled amino acid incorporation, which was taken as a measure of protein synthetic activity, was always found to be appreciably lower in mutant cells. The parental A. indica cells showed a considerable 'dip' in [³H]leucine incorporation at approximately 6 h after mitosis, whereas, no such depression in protein synthetic activity was observed in the mini mutant cells. The mutant amoebae displayed a more or less steady state of labelled amino acid incorporation up to mid G₂ phase, and thereafter there was a dramatic decline in incorporation during the rest (late G₂) of the cell cycle (Fig. 15). In contrast, there was no such inhibition of tritiated leucine incorporation in the corresponding period of the cell cycle of A. indica but there was, instead, a steady increase in [³H]leucine incorporation.

Fig. 15. Pattern of [³H]leucine incorporation throughout the cell cycle of control and mutant amoeba. Each point represents the mean of 42-48 cells from three separate experiments. S, S phase; EG₂, early G₂ phase; MG₂, mid G₂ phase; LG₂, late G₂ phase. Other details and symbols as in Fig. 14.
**Possible mode of origin of mini amoeba**

We became interested in investigating further the possible cell-cycle-phase-specific mode of origin of these mini cells. Routine cytological preparations of the early $S$ phase treated cells were undertaken from 10 h onwards. A striking change in the nuclei of the treated cells could be seen in both the light and the electron microscope. In a few cases we observed the presence of more than one type of nuclear material in the EMS-treated amoebae between 12 and 15 h following treatment. There seemed to have been a premature nuclear division resulting in the formation of smaller nuclear bodies with prominent nucleoli (Figs 16–18).

In a few cases we were also able to detect the emergence of mini cells at around 36 h following the treatment of early $S$ phase cells with EMS. In cytological preparations, it was observed that these mini cells emerged from the treated cells containing a comparatively smaller nucleus along with some portion of the cytoplasm (Fig. 19). The mini cells were ultimately found to be detached from the treated cells. A delay in division of 5–6 days was noticed after their origin from the early $S$ phase treated cells, although, once the division was initiated the cells continued to divide to form a clone of mini amoebae of almost identical size.

**DISCUSSION**

Our experiments were designed primarily to analyse the action of EMS on cells of *A. indica*, a somatic replicating cell type, at various cell cycle stages, and also to discover which phase(s) of the cell cycle were vulnerable to mutation by EMS treatment. EMS acts as a mutagen mainly by the ethylation of guanine at the 7-position (Swann & Magee, 1971). Other possible mechanisms of EMS action have been recorded (Kimball, Setlow & Liu, 1971; Anderson & Fox, 1974; Singer, 1979).

The present observations show that after treatment of amoebae at 1-h intervals throughout the cell cycle the stable mini mutant originates only from early $S$ phase treated cells. The nucleus of amoeba has been considered to be polyploid, though the existence of polyploidy is based on circumstantial evidence (Ord, 1973, 1979c). A mutation in amoebae, which are considered to have many copies of each gene, would

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Fig. 16. An amoeba treated with EMS at early $S$ phase, showing more than one type of nuclear material (arrows) at approx. 14 h after treatment. Squashed and Giemsa-stained preparation. ×1600.

Fig. 17. Thin section of an amoeba treated with EMS at early $S$ phase. Cell fixed at approx. 14 h following treatment and stained with Toluidine Blue. Note three nuclei (arrows) with distinct nucleoli. fo, food vacuole. ×1600.

Fig. 18. Electron micrograph, from a serial section of a portion of an early $S$-phase-treated cell fixed at approx. 14 h after treatment, showing two nuclei ($n_1$ and $n_2$) and part of another nucleus ($n_3$). ×4500.

Fig. 19. Possible origin of a mini amoeba mutant from an *A. indica* treated at early $S$ phase. Cell fixed at approx. 36 h after treatment. Note that the treated parental cell is binucleate and the mini cell has a considerably smaller nucleus. Arrow indicates the point of cytoplasmic separation. ×252.
require a change in all, or at least many, of the copies. When EMS is present for only a brief period of the cell cycle it would be necessary for all the copies of the particular genes to be vulnerable at the same time, while most of the other genes are protected. Two of the activities of chromatin are expected to make genes vulnerable to EMS: DNA replication and DNA transcription. During these periods particular genes are likely to be vulnerable, while in other periods they are expected to be less affected. The expression of inheritable change in a polyploid system, e.g. *A. indica*, might lead to non-random changes in specific cistrons, such as has been shown in amoeba cells (Ord, 1976) and also in other systems (Botstein & Jones, 1969; Guerola, Ingraham & Creda’Olmedo, 1971; Guerola & Creda’Olmedo, 1975) after treatment with *N*-methyl-*N*-nitrosourea.

The early S-phase-dependent EMS-induced mini mutant production in amoebae suggests that the mutagenic action of EMS is primarily confined to the period of DNA replication in which maximum DNA ethylation is possible. It also suggests that the particular genetic location(s) that appear to be affected, and thus responsible for the production of the mutation, are only vulnerable during a short period of DNA replication. The findings of Kee & Haber (1975) using nitrosoguanidine treatment and cell-cycle-dependent induction of mutation along the yeast chromosome, and mutations during the cell cycle phases of hamster tissue-culture cells (Orkin & Littlefield, 1971), as well as induction of mutation in *Paramecium* (Cohen, 1980) with EMS at the DNA replicating phase, strongly support the present findings.

We have tried to investigate critically the probable mode and time of mini cell origin after EMS treatment. It has been observed that around 12—15 h following treatment of early S phase cells, break-up of nuclear material seems to occur at interphase resulting in the formation of small nuclei. At around 36 h after treatment, a portion of the treated parental cell cytoplasm enclosed by plasma membrane has been found to be separated, presumably by an out-of-step cytoplasmic division, and to contain a smaller nucleus. It is quite likely that the mini cell containing the nucleus, which presumably has originated by break-up of the parental nucleus at interphase, may be carrying some altered genetic information. This might lead to the formation of stable clones, after division of mini mutants, with different characteristic features from that of the parental cells. It is suggested that the smaller size of the mini mutant nucleus may be due to balanced elimination and subsequent redistribution of nuclear material during the process of nuclear fragmentation. The viability of mini mutant cells indicates that their nuclei are capable of performing all the functions essential for survival and multiplication. The cell size and nuclear diameter of mini mutants are quite small as compared to *A. indica* and are consistent, which strongly suggests that it is a size mutant. We have failed to detect the existence of any endosymbiont(s) in more than 30 *A. indica* cells examined under the electron microscope.

The cell membrane of the mutant cell has developed a great deal of surface adhesiveness and this has remained a constant feature. Increased adherence to the substratum definitely indicates change(s), structural and probably conformational, in the mutant cell membrane. One of the most important features of the mini mutant is the change in its feeding habits, from *Tetrahymena* to *Chilomonas*. It is likely that in
mini cells the total area of plasma membrane required to capture more than one *Tetrahymena* and form more than one phagosome at a time is inadequate. The observations indicate that *Chilomonas*, being smaller than *Tetrahymena*, is the most suitable food for mutant amoebae. Formation of several phagosomes at a time suggests a 'comfortable' physiological state for mini mutants in relation to *Chilomonas* capture, phagosome formation, ingestion and assimilation of nutrients. It is clear from our scanning electron microscopic studies that there are remarkable differences in the cell surface architecture between mini and parental amoebae. The almost exclusive presence of pits, mini beads and blebs with different structural configurations on the cell surface of the mutants, as compared to the surface of the parental cells, shows the distinct and different structural configuration of the plasma membrane of mutant amoebae.

Among the cytoplasmic organelles, the rough endoplasmic reticulum appears to have become vesicular in mutant cells. In control amoebae the structure of the rough endoplasmic reticulum is in the form of cisternae. This difference in configuration suggests a characteristic structural pattern. The cytoplasmic triuret crystals of amoebae (Griffin, 1961) are found to change their form after treatment with N-methyl-N-nitrosourethane (Ord, 1970). Changes in the crystal form of EMS-induced mini amoeba mutants, as compared to the control cells, have also been noted; they may be due to alteration of nuclear DNA or its closely associated chromatin protein (Ord, 1970, 1979b).

The most striking feature of the mutant cell has been the appearance of a layer of microfibrils around the outer membrane of the nuclear envelope, suggesting the possible involvement of new structural protein(s). The highly condensed and sharp contours of the nucleoli, as compared to those of the control cells, probably indicate a higher degree of intermixing and close packing of granular and fibrillar elements, unlike that found in the parental cells. All these features lead to the conclusion that the nuclear structure of the mini cell is definitely different from that of the parental cell. The origin and significance of the electron-dense particles, observed within the mini cell nuclei, are not clearly understood.

The comparatively shorter duration of *S* phase and the profile of $[^3H]$thymidine incorporation in the nuclei of mutant amoebae strongly suggest the existence of a different pattern of DNA synthesis as compared to the control cells. A comparison between the pattern of tritiated leucine incorporation at different phases of the cell cycle of the control and the mutant amoebae also shows a distinct difference in the profile of protein synthesis, thus indicating a definite functional difference between these two types of cells.

Analysis of the cell cycle of mutant amoebae thus reveals shorter durations of different phases as compared to parental cells, leading to an overall shortening of the cell cycle. Alteration in the cell cycle timing has also been recorded in *N*-methyl-*N*-nitrosourethane-induced amoeba mutants (Ord, 1970, 1973), in which a longer cell cycle was noted in all cases. It has been suggested (Killander & Zetterberg, 1965; Hartwell, Culotti, Pringle & Reid, 1974; Prescott, 1976) that there is a size control over the DNA division cycle. It is probable that attainment of a particular cell size
itself is not the trigger, but perhaps some property that changes with size, such as accumulation of a specific cell substance, leads to faster cell division (Carter & Jagadish, 1978). Nuclear-transfer experiments (Ord & Bell, 1968) between normal and mutant amoebae show the direct action of the mutant nucleus in controlling the length of the cell cycle. Cell cycle progression depends on the consecutive and/or simultaneous action of a series of genes. Malfunction or quantitative change in the action of any of these genes owing to mutation might lead to an alteration in the cell cycle progression. A mutation that, under permissive conditions, alters the cell cycle in a unique way is a cell cycle mutant (Simchen, 1978). The mini amoeba mutant obtained by us, however, does not satisfy all the characteristics necessary to designate it as a cell cycle mutant.

The foregoing discussion strongly suggests that the EMS-induced mini amoeba cell is a size mutant that has a cell-cycle-phase-specific origin, displays a totally altered cell cycle pattern and has a characteristic structural, physiological and biochemical identity.

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Cell cycle mutant of amoeba


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