NUCLEAR MORPHOLOGY AND THE ULTRASTRUCTURAL LOCALIZATION OF DEOXYRIBONUCLEIC ACID SYNTHESIS DURING INTERPHASE

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SUMMARY

The ultrastructural localization of deoxyribonucleic acid (DNA) synthesis was studied by electron-microscope autoradiography in human transforming lymphocytes, embryonic lung fibroblasts, epithelial cells and normoblasts. Euchromatin was found to be active in DNA synthesis in all cell types studied, whereas heterochromatin was inactive. However, DNA synthesis was also prominent in the regions where heterochromatin was thought to be decondensing to form euchromatin. Analysis of sequential changes in nuclear morphology of the transforming lymphocyte suggested that there is decondensation of heterochromatin during the S-phase until none is left. In nuclei with no heterochromatin a prominent localization of DNA synthesis was at the nuclear membrane. This sequence of complete decondensation of heterochromatin also seemed likely for fibroblasts and epithelial cells. Normoblasts however showed no stage where the nucleus was wholly euchromatic and it is suggested that in this cell decondensation of heterochromatin for replication is localized and transient.

INTRODUCTION

The cell nucleus possesses chromatin in two forms, a condensed form, heterochromatin, and a less condensed form, euchromatin. The deoxyribonucleic acid (DNA) concentration of heterochromatin has been shown, by microspectrophotometric measurements of Feulgen-stained nuclei, to be two to three times that of euchromatin (Lima-de-Faria, 1959). The heterochromatic areas which stain densely with the uranyl stain used in electron microscopy are identical with those which appear dense after treatment with the Feulgen stain (Littau, Allfrey, Frenster & Mirsky, 1964).

Autoradiographic studies, with both the light and electron microscopes, suggest that it is the euchromatic areas which are active in ribonucleic acid (RNA) synthesis (Littau et al. 1964; Berlowitz, 1965; Karasaki, 1965; Granboulan & Granboulan, 1965) and also in DNA synthesis (Hay & Revel, 1963; Meek & Moses, 1963). Corroboration of these views comes from incorporation studies on nuclei fractionated into their heterochromatic and euchromatic components (Frenster, Allfrey & Mirsky, 1963).

All the DNA of the nucleus must replicate before cell division. If replication occurs only when the DNA is in the decondensed form, either there must be a stage at which the nuclear chromatin is totally decondensed, or localized areas of heterochromatin
must decondense, replicate, and then recondense during the S-phase. Studies of synchronized cells might be expected to clarify this problem, but it is probable that the most widely used methods of synchronization by metabolic inhibition affect the subsequent DNA metabolism of the cell (Till, Whitmore & Gulyas, 1963; Rao & Engelberg, 1966).

An alternative approach is to follow the changes in the chromatin of lymphocytes transformed by phytohaemagglutinin where the first interphase is delimited by the first mitosis. This study has used electron microscopy combined with autoradiography to follow the changes in nuclear chromatin and sites of DNA synthesis during the first S-phase of the transforming lymphocyte. A preliminary account of these observations has been published elsewhere (Milner & Hayhoe, 1968). Observations made in the transforming lymphocyte have now been extended to other cell types.

**MATERIALS AND METHODS**

**Lymphocyte cultures**

Lymphocytes from 20 ml of peripheral blood from normal male donors were separated by sedimentation with gelatin (Coulson & Chalmers, 1964). Cultures were set up in universal containers in TC 199 containing 200 units/ml penicillin and 100 mg/ml streptomycin and 25% autologous serum. To each 6-ml aliquot 0.2 ml of phytohaemagglutinin (PHA) was added. One vial of Bacto-Phytohaemagglutinin M, Difco, was rehydrated with 5 ml of sterile distilled water. The stationary cultures were incubated with air as the gas phase. At appropriate intervals tritiated thymidine ([3H]thymidine), specific activity 17.7–22.4 c/mmole (obtained from the Radiochemical Centre, Amersham) was added to a final concentration of 5 µc/ml for specified times. The cultures were then terminated.

**Human cell lines in culture**

Human embryonic lung fibroblasts were cultured in Eagle's medium with 10% foetal calf serum and 1% sodium bicarbonate. They were in their fifth transfer at the time of the experiment. Human epithelial cells originally derived from a laryngeal carcinoma (Moore, Sabachowsky & Toolan, 1955) were grown in TC 199 with 2.5% foetal calf serum. Both cultures were kindly supplied by Dr P. Dendy. 5 µc/ml [3H]thymidine were added for 30 min to each culture and these were then terminated.

**Human normoblasts**

Fragments of sternal marrow from two normal donors were incubated in TC 199 and [3H]-thymidine for 1 h as described above. The fragments were then processed for electron microscopy and autoradiography.

**Light microscopy and autoradiography**

Slides for morphology and mitotic counts were stained with Leishman's stain. Slides for autoradiography were coated by the dipping method with Ilford L4 emulsion after staining with periodic acid/Schiff stain (PAS). They were exposed for 2 days, and after development counterstained with haematoxylin.

**Electron microscopy and autoradiography**

The cultures were terminated by fixation in 6.25% glutaraldehyde in 0.15 M phosphate buffer at pH 7.2 for 1 h. After washing in 0.22 M sucrose in 0.15 M phosphate buffer at pH 7.2 they were post-fixed in unbuffered 1% osmium tetroxide for 1 h. Rapid dehydration in a series of alcohols
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and propylene oxide was followed by embedding in Araldite. Sections were cut on an LKB Ultrotome, picked up on carbon-coated grids and stained with uranyl acetate. A second thin carbon film was shadowed on top of the sections and they were then covered with a monolayer of Ilford L4 emulsion by the loop method (George, 1961). After exposure for 3 weeks, grids were developed with Microdol-X (Kodak) and further stained with lead citrate (Reynolds, 1963). They were viewed in an AEI EM6B electron microscope.

Whenever cells were counted in the electron microscope care was taken that different thin sections of the same cell were not included in the count. To ensure this, only one section per grid was counted and as a routine several thick sections were cut between each successive grid.

RESULTS

In common with other workers (Bender & Prescott, 1962; McIntyre & Ebaugh, 1962; Michalowski, 1963; Shapiro & Levine, 1967), it was found that there is negligible DNA synthesis for the first 24 h after initiation of a lymphocyte culture with PHA. Even at 24 h cells synthesizing DNA were scanty, but by 30 h they averaged 6%. [\(^3\)H]Thymidine added to a culture at this time therefore virtually labelled cells only at the beginning or in the first part of their S-phase. The morphology of such cells showed that [\(^3\)H]thymidine is taken up only by cells with nuclei containing less heterochromatin than the parent lymphocyte. Figure 1 shows a cell with the greatest amount of heterochromatin found. It appears therefore that some decondensation of chromatin has taken place before the onset of DNA synthesis. It is also apparent from Fig. 1 that grains appear over areas of euchromatin but there also seems to be a definite localization at the borders of the heterochromatin. This junctional labelling was very prominent in some cells and is better seen in Fig. 2. A number of labelled cells showed the heterochromatin largely confined to a thin band at the periphery of the nucleus and this was often associated with a considerable concentration of grains. At 30 h all labelled nuclei contained heterochromatin although in some it was present merely as a thin marginal rim apposed to the nuclear membrane.

When [\(^3\)H]thymidine was added to a 48-h culture an additional type of cell now showed labelling. These cells had nuclei with no heterochromatin at all. The time of appearance of these cells with wholly euchromatic nuclei was investigated and related to other parameters of the lymphocyte cultures. The percentage of transforming lymphocyte nuclei without any heterochromatin was determined at successive intervals for cultures from two different donors. Three hundred cells were counted in the electron microscope for each time interval from each culture. These results were related to the percentage of cells synthesizing DNA as determined by a count of 500 cells and the mitotic index from a count of 2000 cells from each of 5 cultures. The percentage of cells in DNA synthesis which had a wholly euchromatic nucleus, as shown by electron-microscopic autoradiography, is shown for a single culture where 200 labelled nuclei were counted at each time interval. The results are shown in Table 1.

It can be clearly seen that those cells with a totally decondensed nucleus appeared after DNA synthesis had begun, but before the stage of mitosis was reached.

The marked localization of grains at the borders of the heterochromatic areas in some cells was noticeable as also was labelling in the region of the nuclear membrane.

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in cells without heterochromatin. It seemed possible that some of the variation in patterns of labelling might be due to the length of time for which the label was available. A 10-min pulse of \(^{3}H\)thymidine was therefore given to a 72-h culture of lymphocytes. Two hundred nuclei were classified as possessing heterochromatin or being wholly euchromatic. They were further classified as peripheral, if labelling was confined to the edges of the peripheral heterochromatin or the nuclear membrane if no heterochromatin was present, as central, if only the central parts of the nucleus were labelled, and as mixed, if both patterns of labelling were present. Examples are shown of a cell classified as containing heterochromatin with a mixed pattern of labelling (Fig. 3) and of a wholly euchromatic nucleus with peripheral labelling (Fig. 4). The results of this analysis are shown in Table 2.

Table 1. Changes in nuclear morphology, labelling with \(^{3}H\)thymidine, and mitotic index in lymphocytes transformed with PHA

<table>
<thead>
<tr>
<th>Period of culture, h</th>
<th>24</th>
<th>30</th>
<th>36</th>
<th>42</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclei with no heterochromatin</td>
<td>0</td>
<td>0.2</td>
<td></td>
<td>4.8</td>
<td>8.7</td>
</tr>
<tr>
<td>Cells labelled with (^{3}H)thymidine</td>
<td>0.4</td>
<td>6</td>
<td>16.6</td>
<td>26.2</td>
<td>33.2</td>
</tr>
<tr>
<td>Mitoses</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0.02</td>
<td>0.6</td>
</tr>
<tr>
<td>Labelled cells with wholly euchromatic nuclei</td>
<td>—</td>
<td>0</td>
<td>1.5</td>
<td>8.5</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Results are expressed as means with the range of values in parentheses.

Table 2. Analysis of nuclear pattern and type of labelling with \(^{3}H\)thymidine for 200 nuclei in a 72-h culture of transformed lymphocytes

<table>
<thead>
<tr>
<th>Type of labelling</th>
<th>Peripheral</th>
<th>Mixed</th>
<th>Central</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterochromatin present</td>
<td>68</td>
<td>82</td>
<td>15</td>
</tr>
<tr>
<td>No heterochromatin present</td>
<td>13</td>
<td>15</td>
<td>7</td>
</tr>
</tbody>
</table>

It can be seen that 91% of the nuclei containing heterochromatin have labelling at the border of the heterochromatin and 41% have labelling at this region only. Where no heterochromatin is present, the majority of nuclei have labelling at the nuclear membrane and in 37% this is the sole significant region of labelling.

The finding that there was a period of the S-phase of the transforming lymphocyte where the nucleus was of a totally decondensed pattern initiated a search to see if a similar event also occurred in other cells. In both human embryonic fibroblasts and human epithelial cells it was found that cells taking up \(^{3}H\)thymidine showed both
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the presence and absence of heterochromatin. Labelled nuclei containing no blocks of heterochromatin from a fibroblast and an epithelial cell are shown in Figs. 5 and 6 respectively. In both types of cell the masses of heterochromatin, where present, were always small but there did seem to be a relation of grains to these areas suggestive of the junctional labelling found in the transforming lymphocyte. Normoblasts from human marrow gave different results: as in the other cell types, grains were confined to areas of euchromatin (Fig. 7), but a survey of many grids showed no stage, either labelled or unlabelled, where the nucleus was without substantial masses of heterochromatin.

DISCUSSION

These results confirm previous work which has indicated that the euchromatic areas of the nucleus are those which are active in DNA synthesis (Hay & Revel, 1963; Meek & Moses, 1963; Frenster et al. 1963). The improved fixation conferred by glutaraldehyde has shown that an important site of DNA synthesis is the margin of the areas of heterochromatin.

No significant labelling over heterochromatin was seen. Where heterochromatin was present in small masses or in a thin peripheral ring it was not possible to be certain that grains did not overlie it, but wherever grain size was small, relative to the areas of heterochromatin, the grains were found to be at the border of the heterochromatin. It seems reasonable to suggest therefore that this is a constant phenomenon. The change in nuclear morphology with increasing culture time suggests that transformation of the lymphocyte involves gradual decondensation of heterochromatin, through stages where heterochromatin is present mainly as a diminishing peripheral rim apposed to the nuclear membrane followed by its eventual disappearance. Decondensation during the first 24 h is not accompanied by DNA synthesis. Cells then begin to enter their first S-phase, the initial part of which at least coincides with the final stages of decondensation. Areas active in DNA synthesis are those which are already euchromatic but as new areas of heterochromatin decondense they become active as a template for DNA synthesis at the point of decondensation.

It might be questioned that some of the cells in DNA synthesis at 48 h are in the second cell cycle. However in the present cultures mitoses were very scanty before 46 h. Furthermore, the G1 period in these cells has been estimated as 6 h (Sasaki & Norman, 1966). The majority of cells labelling with [3H]thymidine at 48 h, and in particular the 15 % which have wholly euchromatic nuclei, must therefore be in the S-phase of the first cell cycle.

The time in the S-phase at which the nuclear chromatin becomes totally decondensed remains undetermined. It is possible that this is the stage when all the DNA has replicated. If such is the case the nucleus may or may not retain this morphology through the G2 rest phase until the condensation of the chromosomes in prophase. Since it seems that only decondensed chromatin replicates, the alternative, that this is not the tetraploid stage, might imply further condensation and decondensation before the end of the S-phase.
This pattern of decondensation of chromatin during interphase may be repeated during later cell cycles of the transformed lymphocyte. After 72 h incubation, for example, nuclei with various amounts of heterochromatin are present. Cells in the S-phase at 72 h were found to have a similar range of amounts of heterochromatin as those in the first S-phase, including cells with no nuclear heterochromatin at all. It seems probable that heterochromatin could persist from incomplete decondensation of the chromosomes after telophase as has been found in HeLa cells (Blondel & Tolmach, 1965).

The complexities of the technique of electron-microscope autoradiography make it feasible to analyse only a relatively small number of labelled cells and the conclusions which can be drawn are limited by this fact. Nevertheless it seems probable that the nuclear membrane is an important locus of DNA synthesis in cells which have no readily discernible peripheral rim of heterochromatin. This may represent activity at the end of heterochromatic decondensation as the last remaining layers decondense at the nuclear membrane. Davies (1968) has recently found that in nuclei from several species the microfibrils of the heterochromatin lying near the nuclear membrane are arranged in several ordered layers parallel to the membrane. It seems possible that the high proportion of euchromatic nuclei showing DNA synthesis in this region could in part be due to the time taken for linear replication of these microfibrils parallel to the nuclear membrane. However, the high proportion of wholly euchromatic nuclei showing labelling in the region of the nuclear membrane suggests that this region may have special significance.

Evidence that DNA synthesis at membrane surfaces may be of importance comes largely from bacteria. It has been suggested that the point of replication of the bacterial chromosome is at the cell membrane (Jacob, Ryter & Cuzin, 1966). It has also been found that DNA labelled with a short pulse of thymidine sediments with the fraction of B. subtilis containing the cell membrane (Ganesan & Lederberg, 1965). There have been suggestions that in higher animals, also, attachment of the chromosomes to a structure such as the nuclear membrane might explain the phenomenon of segregation of sister chromatids during mitosis (Lark, Consigli & Minocha, 1966) or the non-random arrangement of chromosomes on the metaphase plate (Sved, 1966; Schneidermann & Smith, 1962). Of interest also is the finding in honey-bee embryonic nuclei that chromatin fibres are attached to the nuclear membrane sometimes in configurations suggesting linear attachment but mainly at the termini of the fibres (DuPraw, 1965).

In summary, therefore, the present results suggest that in the transforming lymphocyte active sites of DNA synthesis are in areas of euchromatin and at the borders of heterochromatic regions as they decondense to become euchromatic. Where no peripheral heterochromatin lies apposed to the nuclear membrane, this is an important locus of DNA replication. In some totally euchromatic nuclei the nuclear membrane region is the sole significant region of such replication; in others more central regions of euchromatin are also active.

Further work will be necessary to establish if this proposed pattern of ultimately complete decondensation of heterochromatin during the S-phase extends to other cell
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types. The finding of completely euchromatic nuclei during the S-phase in human fibroblasts and a human epithelial cell line suggests that it may. On the other hand Blondel & Tolmach (1965) found no such stage in their study of the ultrastructure of the nucleus in HeLa cells synchronized by washing off the non-adherent mitotic cells.

The normoblasts have a nucleus which through several cell divisions becomes gradually more condensed and functionless. Nevertheless it may provide an example of an alternative pattern. The late normoblast contains the diploid content of DNA (Grasso, Woodard & Swift, 1963), thus all the DNA must be replicated at each preceding cell division. Since the present study has indicated that DNA replicates only as euchromatin in this cell type also, and no totally euchromatic nuclei were seen, it seems probable that local areas of heterochromatin must decondense, replicate and then condense again during an S-phase. In keeping with this is the finding that in human normoblasts there is no relationship between minor differences in nuclear structure and the position of the cell in interphase as assessed by its DNA content (Wickramsinghe, Cooper & Chalmers, 1968). Heterochromatin lies apposed to the nuclear membrane over most of its circumference in this cell type and DNA synthesis has been observed to be in more centrally lying euchromatic areas. Normoblasts therefore also differ from transforming lymphocytes in that the region of the nuclear membrane is not a prominent locus for DNA replication.

The basis for the structural difference between euchromatin and heterochromatin remains undetermined. It has been shown that demarcation between the types of chromatin is sharp but there is continuity of microfibrils between them (Frenster, 1965). Removal of the lysine-rich histone fraction from isolated calf thymus nuclei results in a decrease of density of condensed chromatin, as viewed in the electron microscope; readdition of this fraction restores the density (Littau, Burdick, Allfrey & Mirsky, 1965). Furthermore, Whitfield & Perris (1968) have found, also in thymocytes, that both inorganic phosphate and a phosphoprotein cause disappearance of condensed chromatin which reappears on addition of lysine-rich histones. They have postulated that phosphate and phosphoprotein combine with histones and thus affect the interaction of DNA and histone.

The hypothesis that histones act as a mechanism for the repression of DNA has received much support since its original suggestion (Stedman & Stedman, 1950). Removal of histone from isolated chromatin increases its activity as a template for RNA synthesis in vitro (Bonner & Huang, 1963; Allfrey, Littau & Mirsky, 1963; Georgiev, Ananieva & Kozlov, 1966; Paul, 1967). Alterations in the relationship between DNA and histone also affect DNA synthesis (Gurley, Irvin & Holbrook, 1964; Billen & Hnilica, 1964; Schwimmer & Bonner, 1965), although the effects are not as marked as in the case of RNA.

Histones have been implicated in the structural configurations of heterochromatin (Allfrey et al. 1963) and the chromosomes at cell division (Zubay, 1964). Although the role of histones in the day-to-day regulation of individual gene function has been questioned (Paul & Gilmour, 1968) it is possible that histones may provide a mechanism for the more permanent repression of a large part of the genome in a differentiated
It is therefore of interest that the present findings indicate that the structural change from heterochromatin to euchromatin is accompanied by marked activity in DNA synthesis.

It has been suggested that chromosomes are located in definite sites in the interphase nucleus (Mirsky & Osawa, 1961; Comings, 1968) and it is now well established that replication begins simultaneously at several points along a chromosome and furthermore that the regions of initiation are constant through successive generations (for example, Taylor, 1960; Gilbert, Muldal, Lajtha & Rowley, 1962; Hsu, 1964). The present demonstration of the important sites of DNA synthesis may provide some ultrastructural basis for the constancy discernible at the level of the chromosome.

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REFERENCES


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Figs. 1, 2. Electron-microscope autoradiographs of transforming lymphocytes from a 30-h culture labelled with \[^{3}H\]thymidine. Grains are seen in areas of euchromatin and where heterochromatin borders on euchromatin (nu, nucleolus). Fig. 1, \(\times 18000\); Fig. 2, \(\times 25000\).
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Figs. 3, 4. Electron-microscope autoradiographs of transforming lymphocytes from a 72-h culture labelled with \^{3}H\]thymidine. The cell in Fig. 3 was classified as possessing heterochromatin with a mixed pattern of labelling. \(\times 19000\). The cell in Fig. 4 was classified as being euchromatic with peripheral labelling (\textit{nu}, nucleolus). \(\times 23000\).
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Figs. 5, 6. Electron-microscope autoradiographs of nuclei which possess no blocks of heterochromatin labelled with [3H]thymidine (nu, nucleolus). Fig. 5. Nucleus of a human embryonic fibroblast. x 12000. Fig. 6. Nucleus of a human epithelial cell nucleus. x 12000.
Fig. 7. Electron-microscope autoradiograph of two human normoblasts labelled with $[^3H]$thymidine. Grains are localized solely over areas of euchromatin. $\times 18000$. 