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# Conditions Determining Initiation of DNA Synthesis in 3T3 Cells*

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**Abstract.** Experiments were designed to discriminate between inhibition of growth due to contacts or exhaustion of serum factors. The cell layer was wounded and the migrating cells were followed by time-lapse cinematography; DNA synthesis in the same cells was recognized by means of $^3$H-thymidine labeling and radioautography. In this way, the complete history of individual cells migrating to the wound could be described. The results show that topographical relationships between cells play an important role in controlling initiation of DNA synthesis. It is still unclear whether initiation is promoted by release from contacts or by the increased ability of the cells to utilize serum factors because of their changes in shapes and activities.

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**Introduction.** Inhibition of both cell movement and growth have been observed in confluent cultures of animal cells: this has led to an interest in the interrelationship of these phenomena, and in the role of cell topography and particularly cell contact. 3T3 cells[1] which have been extensively studied by Green and his colleagues, are highly sensitive to growth inhibition in confluent cell layers. Moreover, Todaro *et al.*[2] have shown that, in artificial wounds made by scratching the layer, a high proportion of cells which migrate into the bare area showed autoradiographic evidence of newly induced DNA synthesis. Similar results were obtained with other cells.[3] This type of wound experiment has, therefore, been used by us to study the interrelationship of growth, movement, and cell topography.

The factors which influence the initiation of cell growth, and perhaps also movement, are likely to occur sometime before the observed event. We have therefore analyzed the prior history of cell entering DNA synthesis by use of time-lapse cinematography combined with autoradiography for thymidine incorporation.

Since it has been shown that serum factors will initiate DNA synthesis even in confluent cultures, the experiments were performed in cultures in which the medium had not been changed for several days after reaching confluency, and in which serum factors were partly depleted.

**Methods.** Confluent cultures of 3T3 cells were prepared with 4 to 6 $\times$ $10^5$ cells/50 mm Nunc plastic Petri dish in enriched Eagle's medium supplemented with unheated 10% calf serum. The medium was normally left unchanged on confluent cultures for 2–3 days before each experiment. Scratches were made by drawing a bent Pasteur pipette across the cell sheet to clear an area 500–1000 $\mu$m in width. Sometimes half the

area of a culture was cleared by drawing a razor blade across the cell sheet. Where necessary, orientation marks were scratched on the outside of the plastic dish under the wound to allow identification of the time-lapse microscopic field in subsequent autoradiographs. For time-lapse cinematography, Falcon tissue culture dishes with depressed lids were used (Cooper dishes) to accommodate the ×6.3 phase contrast objective of a Zeiss microscope.

Time-lapse cinematography was performed with the Sage model 152, incorporating a 37°C gassed chamber with humidified $CO_2$ in air (adjusted to pH 7.4 in the culture). The conditions for filming were: film: Kodak plus X reversal; flash intensity: 4–8; speed: 1–2 frames/min. Films were analyzed by tracing individual cells and their paths on a transparent sheet superimposed on a projected image.

For autoradiographs, with or without time-lapse cinematography, 5 μCi of $^3$H-thymidine was added for the appropriate time. Cultures were then washed with Tris-saline, fixed in cold 5% trichloroacetic acid, washed in ethanol, dried, and then exposed for 2–3 days to Kodak AR-10 stripping film added directly to the plastic surface. After exposure and development, cultures were stained with 10% Giemsa.

Total and labeled cells in the final autoradiographs were analyzed by counts in rectangular microscope fields, 450 μm long and 100 μm wide, arranged with the longer sides parallel to the edge formed by the scratch in the confluent cell sheet. This gave the proportion of labeled cells at 100-μm intervals moving across the edge of the sheet.

**Procedures and Results.** In several experiments, continuous films were made showing the edge of a confluent sheet and the scratch gap for up to 48 hr after wounding. Most of the migrating cells were traced and their speed and direction of movement analyzed. In the undisturbed confluent sheet more than about 100 μm from the edge, the majority of the cells displayed slow oriented movement (a few micrometers per hour). In the confluent sheet close to the edge, cells within five cell rows from the edge moved faster, occasionally about 10 μm/hr, with general orientation toward the cell-free area. This oriented mass movement continued up to a distance of about 100 μm from the original edge, "proximal invaded zone," the cells remaining in partial reciprocal contact. Beyond this zone, in the "distal invaded zone," the cells usually moved more rapidly (from about 10–15 to 60–80 μm/hr at times), independently and randomly, except that they were diverted in casual collisions with one another owing to contact inhibition[4] (Fig. 1).

**Thymidine incorporation:** Autoradiography, performed after exposure to $^3$H-thymidine, showed incorporation in a high proportion of cells which had migrated into the denuded area. Figure 2 shows the distribution of labeled cells in the wound. The highest incorporation was in the free cells of the distal invaded zone.

In another experiment, replicate cultures were wounded and exposed to pulses of thymidine for successive 4-hr periods. The proportions of labeled cells were then determined by counting cells in three different zones: the original layer, the proximal invaded zone, and the distal invaded zone (Fig. 3). In the confluent original layer, incorporation was low at any time and declined toward the end of the experiment, probably because of exhaustion of serum factors (since the medium was not changed or replenished during the experiment). Thymidine incorporation increased 12–16 hr after creation of the edge and occurred in migrating cells located mostly in the proximal invaded zone, but some in the distal invaded zone. After 16 hr, incorporating cells were localized mostly in the distal

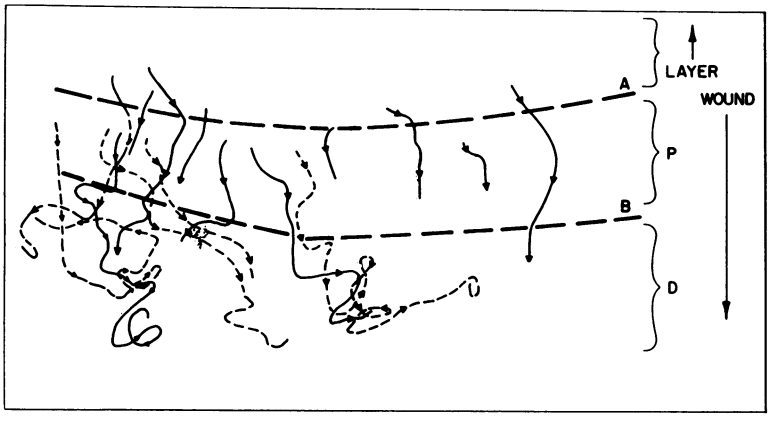


FIG. 1.—Tracings of paths of a number of cells migrating over a period of 32 hr. Different symbols are used only to allow identification of the different paths. *Line A*: original edge of the wound. *Line B*: limit between proximal invaded zone (*P*) and distal invaded zone (*D*). Different path symbols are used only for clarity.

invaded zone. In the whole experiment, the average time before DNA synthesis was 20 hr. Toward the end of the experiment, the proportion of labeled cells decreased, probably because of serum exhaustion. The decline was more marked in the proximal invaded zone, although its cell density remained constant by definition (see legend to Fig. 3).

This experiment showed that DNA synthesis occurred in cells which migrated, irrespective of distance traveled.

**Relationship of movement, topography, and DNA synthesis:** With a combination of autoradiography and time-lapse cinematography it was possible to study the history of each cell in the filmed areas from the moment of wounding on. The initiation of DNA synthesis in a given cell could thus be correlated with certain features of the past history of the cell. The following correlations were examined.

**(*a*) Initiation of DNA synthesis in relation to cell location immediately after wounding:** Many of the cells were located at the free edge of the scratch after wounding, but this was not a requirement since also cells located several rows

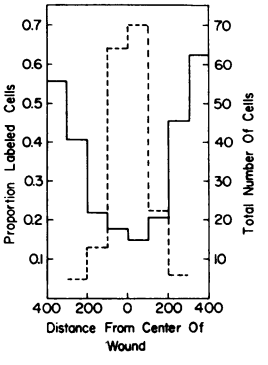


FIG. 2.—Distribution of total (—) and radioactive (- - - -) nuclei in a wound. The proportion of incorporating nuclei is given on the left scale, the number of total nuclei per 100 × 450 $\mu$m fields is on the right scale. Labeling with $^3$H-thymidine was between 17 and 34 hr after wounding, which was during the first cycle of incorporation in the wound.

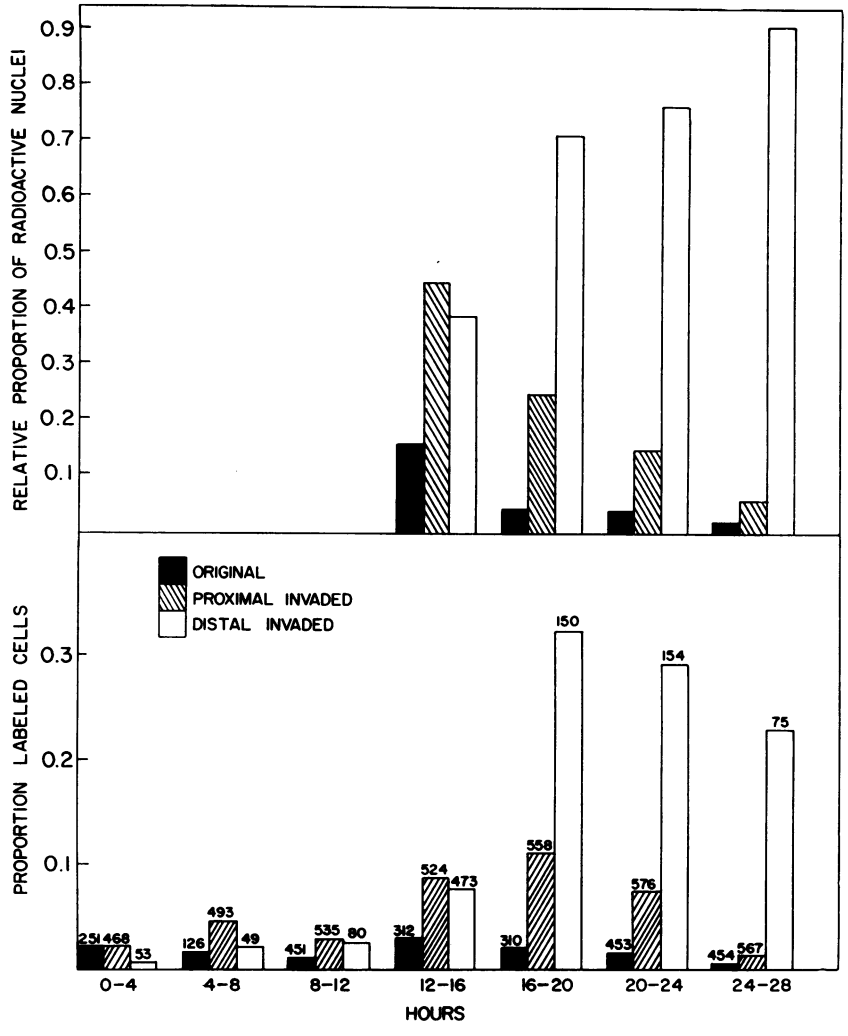


FIG. 3.—Distribution of proportions of radioactive nuclei in 100 × 450 μm fields in the various zones (*original layer*, *proximal invaded zone*, and *distal invaded zone*) during a 4-hr $^3$H-thymidine incorporation at various times after wounding (*lower graph*). *Upper graph:* same proportions as above but normalized to a total of unity in each labeling regime. Total number of cells counted are given but these do not bear any relation to cell density. The original layer contained 20–50 cells per field; the strip where cell density was 18–20 cells per field was taken as the proximal invaded zone. The distal invaded zone contained 1–10 cells per field.

behind could do so, as shown in Figure 4. Furthermore, some DNA-synthesizing cells entered the filmed area sometime after wounding and came, therefore, from even deeper locations.

**(*b*) Relationship to characteristics of cell movement:** The distance covered after wounding by DNA-synthesizing cells along the migration paths varied greatly between 100 and 500 μm without any obvious difference between synthesizing and nonsynthesizing cells. The speed of motion appeared also unrelated to initiation of DNA synthesis.

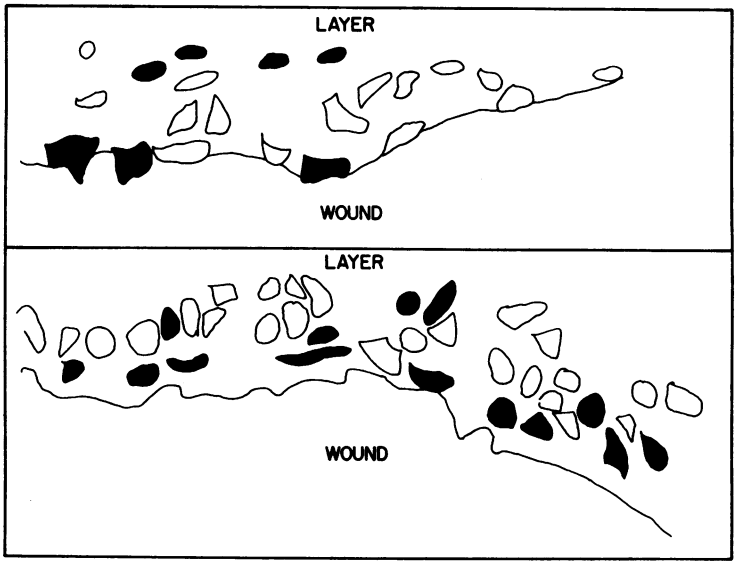


FIG. 4.—Location of some cells shortly after wounding, indicating those incorporating DNA 20–26 hr after wounding in two different experiments. Incorporating cells, solid; nonincorporating cells, empty.

**(c) Relationship to contacts with other cells:** Most of the DNA-synthesizing cells were free from contacts with other cells at the time of synthesis (20–26 hr after wounding). The contact history was determined for 35 synthesizing cells and 24 nonsynthesizing cells (mostly also free), in three different experiments. We determined the time of "release" during which a cell did not enter into contact with other cells over more than half of its perimeter as observed in the film. The results given in Figure 5 show that essentially all the cells examined were

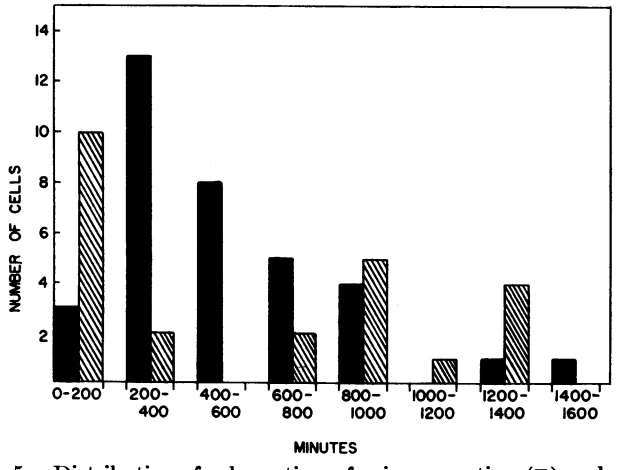


FIG. 5.—Distribution of release times for incorporating (■) and nonincorporating (▨) cells. Pooled data of three experiments. Labeling 20–26 hr after wounding in two cases, 23–26 hr in the third case.

"released" for some time, but there were differences between synthesizing and nonsynthesizing cells. Synthesizing cells had a unimodal but wide distribution of release times with an average of 8.6 hr; nonsynthesizing cells had a bimodal distribution with averages of 2.25 and 17 hr, respectively.

**(*d*) Behavior of postmitotic cells:** We followed the progenies of mitotic cells whenever possible. When both daughter cells entered the wound, they behaved equally with respect to DNA synthesis, i.e., they were both scored either as synthesizing or nonsynthesizing cells. In two cases in which a second round of mitosis was seen, the two daughter cells entered mitosis within less than an hour from each other. As expected, the two cells emerging from mitosis tend to behave similarly during the subsequent division cycle. In three instances, one of the two postmitotic cells went into the wound while the sister cell went into the cell layer, and both could be followed to the end. In one such instance, neither of the two daughter cells showed DNA synthesis, but in the other two, the partner in the wound synthesized DNA whereas the one in the layer did not. In all cases, the grain density over the synthesizing partner was high, showing that synthesis had not just begun at the time the experiment was terminated. We conclude that initiation of DNA synthesis is determined subsequent to mitosis.

**Discussion.** In considering the factors determining the initiation of DNA synthesis in a particular cell in the wound, it is essential to consider events in the prior history of that cell. Formally, initiation could result either from a single activating event at the time of wounding, or from the accumulation of numerous events at successive times after wounding. We cannot make a clear-cut choice between these two hypotheses, although the latter one seems more likely, in view of the different fate of postmitotic cells, depending on whether they move to the wound region or back to the cell layer.

Although DNA synthesis was occasionally observed in contacting cells of the proximal invaded zone, partial or total release of the cell from contact with neighboring cells during its prior history is probably an important factor in initiating DNA synthesis. In fact, the time interval between mitosis and onset of DNA synthesis was about 7 hr in completely released cells, $8^1/_2$ hr (on the average) after release from contact (see Fig. 5), but 20 hr (on the average) after wounding (Fig. 3). It is likely that the time difference in the wound compared to the postmitotic cells arises from a requirement for release. Furthermore, there is an optimal length of the release time for DNA-synthesizing cells, as shown by the distributions in Figure 5; in cells with very long exposure time, DNA synthesis was presumably initiated earlier and had already terminated at the time of labeling. In different synthesizing cells, the previous exposure periods vary very markedly (beyond the range allowed for by the length of labeling time and of DNA synthesis), suggesting that other factors are simultaneously involved, perhaps involving serum factor uptake.

These experiments emphasize the importance of topographical factors in determining the initiation of DNA synthesis in 3T3 cells. Concerning the nature of these factors, two alternatives can be proposed. Either cell-to-cell contact

has a direct inhibitory effect, perhaps involving specific interaction sites, or it inhibits indirectly, e.g., by occlusion of receptor sites for uptake of growth factors. The first alternative would correspond to "contact inhibition of growth," to which there is abundant reference in the literature, but for which conclusive experimental evidence is lacking. The second alternative would leave the main control of cell growth to humoral factors present in the medium.

To explain our observations, growth factor supply and topography would act in a quantitative way (e.g., cell-to-cell contacts on a small part of the cell surface might be less inhibitory than contact over the greater part of the surface). Thus, for an individual cell, the time required before initiation of DNA synthesis would result from the time integral of growth factor concentration and topography, and could vary widely in relation to differences in historical details. This would explain the wide distribution of release times, the fact that neither movement itself, nor distance traveled, can determine initiation, and the occasional initiation of DNA synthesis in cells of the proximal invaded zone, although they still have frequent contacts with other cells.

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