FORMATION OF POLYRIBOSOMES DURING RECOVERY FROM CONTACT INHIBITION OF REPLICATION

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Previous studies (3) of colony formation of 3T3, a cell line with the property of contact inhibition of replication, demonstrated a coincidence of cell contact and cessation of DNA synthesis and replication. Also, plating efficiency tests (3, 6) from monolayer cultures of 3T3 consistently have been in the range of 20%–30% for exponentially growing cells as well as cells in the state of contact inhibition of replication (CI culture), whether in fresh medium or medium from the CI culture. This suggested that the inhibition of macromolecular synthesis and replication was a reversible and inherent property of the 3T3 cell rather than a depletion of the medium or physiological condition of the cells.

A temporary release of the CI culture can be achieved either by dispersing the cells so that they are no longer in contact with other cells or by changing to fresh medium (5). Todaro et al. (5) showed that immediately after a change of medium the cells of a confluent culture underwent a wave of RNA synthesis and that a small fraction of cells eventually completed DNA synthesis and mitosis. The discovery that saline medium without fresh serum could also elicit a stimulation of RNA synthesis (6) led to the hypothesis that an inhibitor of cellular replication exists in confluent cultures of 3T3. In that study the evidence suggested that confluent cultures of 3T3 released a diffusible substance which enabled them to sustain a state of contact inhibition of replication. The site of action of the low molecular weight inhibitor was related to cellular RNA synthesis. In this report we have examined the effects of contact inhibition of replication on the polyribosome (polysome) content in the cell line 3T3.

MATERIALS AND METHODS

The origin and growth characteristics of the cell line 3T3 have previously been described in detail (3, 6). Test cultures were prepared by inoculating either 2×10^5 cells into 2.0 ml of fresh growth medium in 35 mm plastic Petri dishes or 2×10^6 cells into 10.0 ml in 100-mm dishes and incubating in 5% CO_2 at 37°C. This procedure produced cultures in the state of contact inhibition of replication (CI cultures) (6) in less than 5 days.

Macromolecular synthesis was measured by adding 0.05 ml of 40 μCi/ml ^{3}H-uridine or ^{3}H-l-leucine in saline to test cultures, incubating for various times up to 4 hr, and then measuring the radioactivity insoluble in cold 10% trichloroacetic acid with a liquid scintillation counter.

Ribosomes in the form of polysomes and monosomes were isolated by using a modification of Flessel's (4) method for protoplasts from which chloramphenicol was omitted. The cell lysate was layered on a 25.0 ml 15–30% linear sucrose density gradient and centrifuged at 50,000 *g* for 3 hr. The gradient was then fractionated into 1.0 ml fractions and the absorbance at 2600 A was read on a spectrophotometer or the radioactivity was determined as described above. Such an analysis shows an identical profile of the 80 S monosomes and the polysomes, whether the measurements are of the absorbance or the radioactivity. Mild degradation with ribonuclease removes the polysome fraction, with a concurrent increase in the monosome peak. The per cent polysomes from area measurements was defined as the per cent of the total area represented by the fractions greater than 100 S. For an exponentionally growing culture, this was about 55%.

RESULTS

When the polysome content was examined as a function of culture age, it decreased at 2×10^4

cells/cm², the cell density at which contact inhibition of replication begins to drastically reduce the RNA synthesis (6). At a density of 4×10^4 cells/cm² the polysome content was down to 23% and remained at this low level. Cultures could be maintained at the depressed polysome level for several days without loss of viability as measured by plating efficiency, and the medium from the CI cultures gave as high a plating efficiency as fresh medium, suggesting that it did not have any nutritional deficiency.

Fig. 1 shows that when a CI culture was changed to fresh medium with 10% calf serum (released CI culture) the polysome content rapidly increased 2.5-fold, reaching the same level as that of normally growing cultures in less than 2 hr. This change took place without any apparent increase in the profile area, suggesting that it might not involve synthesis of new structures but only an assembly of the monosomes already present. These results led to the study of the effects of metabolic inhibitors on the recovery.

Actinomycin D inhibits transcription and was used to study the role of transcription in the re-formation of polysomes and in protein synthesis in released CI cultures. Test cultures were inoculated with actinomycin D (Merck, Sharp & Dohme, West Point, Pa.) at 12.5 μg/ml (a concentration corresponding to 96% inhibition of RNA synthesis) before a change to fresh serum medium containing the same concentration of inhibitor, and the cultures were assayed for protein synthesis at various time intervals. The results showed that the rate of protein synthesis as measured by ^{3}H-leucine incorporation did not increase as in control cultures which were not subjected to the actinomycin D, and that there was no evidence of release from contact inhibition of replication. Instead, protein synthesis continued as though the cells were still inhibited, suggesting that RNA synthesis was required for the recovery of protein synthesis normally initiated by the release of CI cultures.

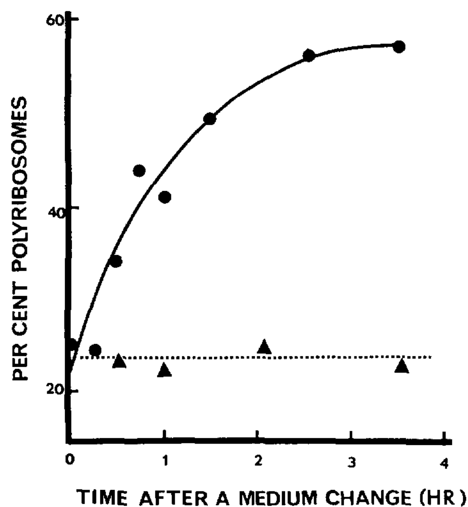

FIGURE 1 Polyribosome content as a function of time after release from contact inhibition of replication. 5-day-old CI cultures were released by a change to fresh serum medium and assayed for polyribosome content after various time intervals. Solid circles (●), fresh medium change (control); solid triangles (▲), fresh medium change plus 12.5 μg/ml of actinomycin D.

Since protein synthesis continued in the presence of the drug for at least 4 hr, it was assumed that the cells were still viable and that the drug was not interfering with translation or binding of ribosomes to messenger RNA to form polysomes. It should be noted, however, that the rate of protein synthesis in the CI culture was about 50% of the normal rate, and that the actinomycin D did not reduce the rate more than that.

The polysome content as a function of time after change to fresh medium in the presence of actinomycin D is also given in Fig. 1. This figure shows that, when CI cultures of 3T3 were released in the presence of a drug that inhibits RNA synthesis, there was no significant increase in the polysome content. Control cultures in which no drug was present showed the expected increase to 55%. This also is suggestive of the need for RNA synthesis for the polysome regeneration.

DISCUSSION

Yeh and Fisher (6) have demonstrated an inhibitor of RNA synthesis in contact inhibition of replication and have suggested that the controlling mechanism is at the point of RNA synthesis. The results of the present investigation are consistent with this interpretation based on the studies with an inhibitor of RNA synthesis. These results further suggest that two levels of biosynthetic capacity may exist in the cell: one to maintain viability, and one for replication. Since the rate of protein synthesis decreased only to approximately half that of normally growing cultures and since this level was unaffected by the inhibitors of RNA synthesis, it appears that there may be a species of

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stable messenger RNA that exists possibly to maintain cell viability.

It is interesting to compare the results obtained in this investigation with the results in previously published reports on similar polysome losses in cultures deprived of nutrients or of essential amino acids (1, 2). Although the mechanism(s) by which both protein synthesis and polyribosome formation are controlled in the case of contact inhibition of replication appear to be dependent on RNA synthesis, such is not the case with either nutrient depletion or amino acid starvation, where it was found that, even if all DNA-dependent RNA synthesis was inhibited, the polysome content increased when the nutrients were returned. This suggests that, in that case, both previously formed messenger RNA and ribosomes were used in the re-formation of polysomes.

SUMMARY

The results of this study indicate that: (*a*) CI cultures had more than a 2-fold reduction in the cellular polysome content and rate of protein synthesis and a 20-fold reduction in the rate of RNA synthesis even though no nutrient deficiency was observed. (*b*) When the CI culture was released by a change to fresh medium, the polysome content increased to the same level as that of normally growing cultures in less than 2 hr. (*c*) The increase in the polysome content during recovery was prevented by the presence of actinomycin D. (*d*) When the CI culture was released by a change to fresh medium, the rate of protein synthesis increased and this increase was also inhibited by actinomycin D.

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