Changes in Template Activity and Structure of Nuclei from WI-38 Cells in the Prereplicative Phase†

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ABSTRACT: Quiescent confluent monolayers of WI-38 fibroblasts were stimulated to proliferate by either adding 10% fetal calf serum or by trypsinization and replating at lower density. The length of the prereplicative phase was 12 hr after serum stimulation and 18 hr after trypsinization and replating at lower density. Nuclei were isolated from WI-38 cells at different time intervals after either type of stimulation and their template activity, circular dichroism spectra, and ability to bind ethidium bromide were investigated. All these parameters were similarly increased after either type of stimulation. However, these changes, like the onset of DNA synthesis, were delayed 6 hr in cells trypsinized and replated at lower density. While there were no detectable changes in nuclear protein content after serum stimulation, at least 40% of nuclear protein, mostly nonhistone chromosomal proteins, were lost after trypsinization. The amount of nuclear proteins returned to prestimulation levels only 6–8 hr after replating. These data seem to suggest that nonhistone chromosomal proteins lost by trypsinization are essential for the entrance of WI-38 cells into the "prereplicative phase".

Quiescent confluent cultures of fibroblasts can be stimulated to divide by two different methods. The first method involves a nutritional change, for instance, the addition of 10% fetal calf serum with or without replacement of the old medium by fresh medium (Todaro et al., 1965). The second type of stimulation involves trypsinization of quiescent monolayers followed by reseeding at a lower density in 10% fetal calf serum with fresh medium (Moscona et al., 1965). These two methods have also been reported to be effective in stimulating WI-38 human diploid fibroblasts (Wiebel and Baserga, 1969; Ellem and Mironescu, 1974; Maizel et al., 1975).

An increase in RNA synthesis is noticeable within the first hour after serum stimulation, as shown by using either isolated chromatin with an exogenous RNA polymerase (Farber et al., 1971) or nuclear monolayer preparations with their own endogenous RNA polymerase (Bombik and Baserga, 1974).

The use of chromatin in template activity studies has been criticized both for the use of an exogenous RNA polymerase (Reeder, 1973) and the method of preparation of chromatin (Comings and Tack, 1973). The technical difficulties in handling trypsinized and newly replated cells in the nuclear monolayer technique also argue for some new methodology. For these reasons, we have isolated nuclei from confluent monolayers of WI-38 cells at various times after serum stimulation or trypsinization and replating at lower density. We have investigated these nuclei in terms of template activity, circular dichroism spectra, ability to bind the intercalating dye ethidium bromide, and protein content.

The results reported in the present paper indicate that the nuclear changes are similar with both methods of stimulation. However, the nuclear changes, like the onset of DNA synthesis, are delayed 5 hr in trypsinized cells replated at a lower density. The differences between serum-stimulated cells and cells trypsinized and replated at lower density suggest that these two methods can be profitably used to investigate the relationship of nuclear proteins to template activity and cell proliferation.

Materials and Methods

Cell Culture. WI-38 human diploid fibroblasts were obtained at passages 14 to 20 from Dr. Leonard Hayflick of Stanford University (through Contract No. NO1-HD-4-2828). Occasionally, the cells were purchased from Flow Laboratories (Rockville, Md.). Cells were mycoplasma free when received. The cultures were monitored periodically for mycoplasma contamination by the autoradiographic technique of Studzinski et al. (1973). Cell stocks at passages 15 to 20 (subcultivation at 1:2 split ratio) were maintained in roller vessels (growth area 840 cm^2) in Eagle's basal medium supplemented with 10% fetal calf serum, 2 m*M* glutamine, and 50 μg/ml of streptomycin. For experiments, cells were grown in 1-l. Blake bottles or plastic Falcon flasks or small petri dishes (surface area 210, 75, and 8.6 cm^2, respectively), at a constant inoculating density of 2×10^4/cm^2. Using the "thymidine incorporation assay" technique of Cristofalo and Sharf (1973), 80–85% of the cells used in the experiments were labeled. This indicated the cells were at the 21–25 cumulative population doubling level (PDL) (Smith and Hayflick, 1974).

Trypsinization and Replating of Cells. The procedure of Maizel et al. (1975) was followed. Cells in monolayers (8 days after plating) were incubated for 1 min with 0.25% trypsin solution (1:250, Microbiological Associates). The trypsin solution was then removed and the cells were placed in an incubator for 10 min. The detached cells were then resuspended in fresh Eagle's basal medium supplemented with 10% fetal calf serum and replated at a density of 2.0×10^4 cells/cm^2.

Autoradiography. For these experiments only, cells were seeded at a density of 2×10^4 per cm^2 on coverslips in small

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petri dishes (surface area 8.6 cm^2). After 8 days, they were stimulated with fresh medium containing 10% fetal calf serum or by trypsinization and replating as described above.

In both cases, 0.1 μCi/ml of [^{3}H]thymidine was added at 0 time. At various time intervals after serum stimulation or trypsinization and replating, six coverslips in petri dishes were rinsed three times with buffered balanced salt solution, fixed, and autoradiographed according to the procedure of Baserga and Malamud (1969). The autoradiographs were analyzed microscopically by scoring the percentage of cells with labeled nuclei in random fields throughout the coverslips. At least 1000 cells were counted on each coverslip. Unstimulated confluent monolayers served as controls and were labeled with [^{3}H]thymidine for 24 hr.

Isolation of Nuclei. (1) Isolation of nuclei for template activity and spectropolarimetric studies. The procedure of Marzluff et al. (1973) was followed with slight modifications. All operations were carried out at 4°. The culture medium was decanted and the cell culture surface rinsed with Ca^{2+} and Mg^{2+} free Hank's (CMFH) balanced salt solution. The cells were then scraped from the bottles with a rubber policeman in CMFH balanced salt solution. The cells were spun down in an International PR-6 centrifuge at 2000 rpm for 5 min and washed two more times with CMFH balanced salt solution. The final pellet was resuspended in 0.25 *M* sucrose, 10 m*M* Tris-HCl (pH 7.5), 2 m*M* $MgCl_2$, and 0.5 m*M* dithiothreitol. This suspension was then homogenized with a glass Dounce homogenizer. An equal volume of the same sucrose buffer with 1% Triton X-100 was added to the broken cell suspension. After vortexing gently, the solution was left standing on ice for 5 min. It was then spun at 2200 rpm for 4 min. The pellet was again washed twice with homogenizing medium. The final nuclear pellet was resuspended in the proper solution for RNA synthesis assay or spectropolarimetric measurements.

(2) Isolation of nuclei for nuclear protein/DNA ratio. For chemical analysis of nuclei at different time intervals after stimulation, clean nuclei free of cytoplasmic contaminations are required. Of the methods for isolating nuclei we tested the procedure of Goto and Ringertz (1974) using Brij 35 seemed to give the most satisfactory results with WI-38 cells. For this technique, the cells were grown on plastic Falcon flasks and the procedure of Goto and Ringertz was followed. The nuclei isolated this way, although they could not actively incorporate [^{3}H]UTP into RNA, had minimal cytoplasmic contamination (see Goto and Ringertz, 1974). The protein/DNA ratio of nuclei isolated from untrypsinized cells was 3.7 (Table I) which is comparable with those of purified nuclei with negligible cytoplasmic contamination isolated from other mammalian cells (Busch, 1967).

Determination of DNA, RNA, and Protein Concentration. For the assay of nucleic acid contents of the nuclei, the procedure of Scott et al. (1956) was followed. For assaying the protein concentration of the nuclei, the lipid-free nuclear residue was solubilized in 1 *N* NaOH and the protein content determined by the method of Lowry et al. (1951).

RNA Synthesis in Isolated Nuclei. The procedure of Marzluff et al. (1973) was followed. The nuclei freshly isolated were resuspended gently at a concentration of about 0.25 mg of DNA/ml in 25% glycerol (v/v), 5 m*M* magnesium acetate, 50 m*M* Tris-HCl (pH 8.0), 1.5 m*M* dithiothreitol, and 0.1 m*M* $EDTANa_2$ (Reeder and Roeder, 1972). Aliquots of 50 μl were taken from the nuclear suspension and mixed with an equal volume of reaction solution containing 5 m*M* magnesium acetate, 2 m*M* $MnCl_2$, 0.8 m*M* each of ATP, GTP, and CTP, 0.1 m*M* of [^{3}H]UTP (specific activity 0.5 Ci/mmol), and 0.3 *M* KCl. The incubation temperature was 37°. The reaction was terminated at various time intervals by adding 5 ml of ice-cold 10% Cl_3CCOOH and 40 m*M* pyrophosphate. The solution was filtered through glass fiber paper (GF/C, Whatman). This was followed by four successive washes of 5 ml of ice-cold 10% Cl_3CCOOH and 40 m*M* pyrophosphate. The filter was then placed in a scintillation vial and incubated with 1 ml of protosol (NEN) at 60° for 1 hr. This was mixed with 15 ml of Econofluor (NEN), shaken vigorously, and counted in a Packard liquid scintillation counter (Model 2425).

Table I: Nuclear Protein/DNA Ratio in Nuclei of WI-38 Cells Stimulated to Proliferate Either by Serum or by Trypsinization and Replating.[a]

Time after Stimulation	Nuclear Protein/DNA Ratio: Cells Stimulated by Changing Medium	Nuclear Protein/DNA Ratio: Cells Stimulated by Trypsinization and Replating
Unstimulated controls	3.3	
1 min	N.D.	1.6
2 hr	3.6	1.9
4 hr	3.7	1.8
6 hr	3.4	2.9
8 hr	3.7	3.2

[a] Confluent monolayers of WI-38 cells were stimulated either by 10% serum in fresh medium or by trypsinization and replating. At different time intervals, cells were collected with a rubber policeman and nuclei were prepared with Brij 35, following the procedure of Goto and Ringertz (1974). The DNA and protein ratios of the nuclei were determined as described under Materials and Methods.

Circular Dichroism of Nuclei. Circular dichroism (CD) spectra of WI-38 nuclei were obtained with a Jasco Model J40 recording spectropolarimeter with CD capacity only. The instrument was standardized as described by Simpson and Sober (1970). All measurements were carried out at 23° in a nitrogen atmosphere using 0.5-cm pathlength fused quartz cells. Light scattering was empirically subtracted on the assumption that the measure of light scattering at 300 nm remained constant between 300 and 250 nm. In agreement with Olins and Olins (1972) the influence of light scattering under our operating conditions did not appear to be great.

Measurement of Ethidium Bromide Binding by Circular Dichroism. Measurements were made on clean nuclei (Maizel et al., 1975) in 0.01 *M* Tris-HCl (pH 8) after removing Mg^{2+}, which decreases the binding of ethidium bromide to nuclei and to DNA (Olins and Olins, 1972). The conditions used were those described by Dalgleish et al. (1971) and by Williams et al. (1972), i.e., a concentration of 140–220 μ*M* DNA-phosphate, and a dye/DNA-P ratio of 0.3. A Jasco J40 spectropolarimeter was used, and the ellipticity was expressed in deg cm^2/dmol of ethidium bromide, assuming a mol wt of 394.

Materials. All radioactive isotopes were purchased from New England Nuclear Corporation. Chemicals, when available, were of reagent grade.

Results

DNA Synthesis in WI-38 Cells Stimulated by Serum or by Trypsinization and Replating at a Lower Density. When quiescent confluent monolayers of WI-38 cells were stimu-

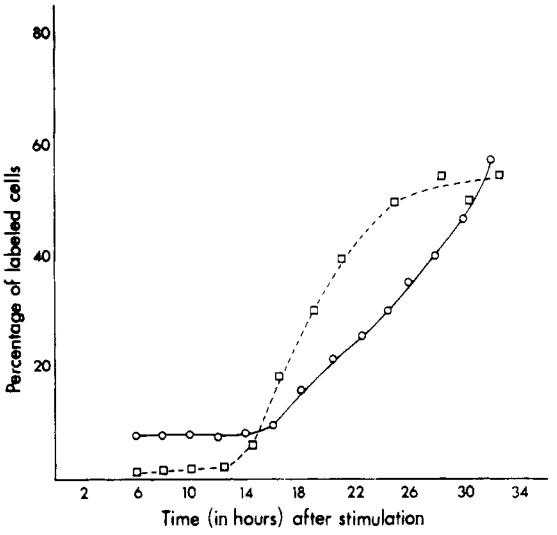

FIGURE 1: Percentage of cells labeled by [^{3}H]thymidine after stimulation of confluent WI-38 cells by changing medium with or without trypsinization and replating. [^{3}H]Thymidine was added at zero time and the percentage of labeled cells was determined by autoradiography as described in the text: (O—O) cells trypsinized and replated at a lower density in stimulation medium; (□- - -□) cells stimulated by changing medium with 10% fetal calf serum in fresh medium.

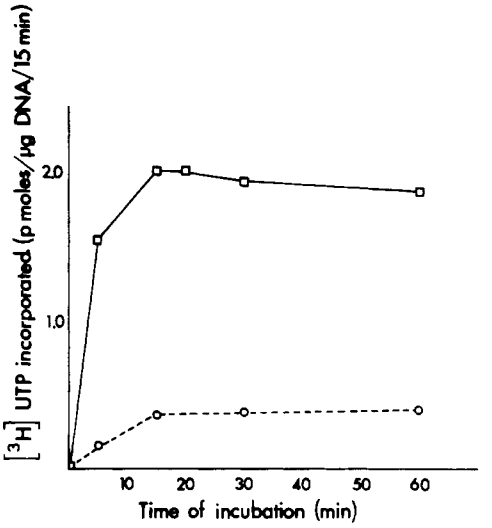

FIGURE 2: Incorporation of [^{3}H]UTP into RNA by isolated nuclei. Aliquots were taken from nuclear preparations and placed into different test tubes with an equal volume of reaction mixture. The incubation temperature of the reaction was 37°. At various times, one or two tubes were taken and the amount of incorporation was determined as described in the text. The concentration of DNA in the nuclear suspension was determined by the diphenylamine assay: (□—□) incorporation of [^{3}H]UTP into RNA in the presence of all four nucleoside triphosphates; (O- - -O) incorporation of [^{3}H]UTP into RNA in the absence of the other three nonradioactive nucleoside triphosphates. It was estimated that 400 cpm is equivalent to 1 pmol of [^{3}H]UTP in our experimental conditions.

lated to proliferate by serum, a wave of DNA synthesis, as determined by autoradiography, began at 12–14 hr poststimulation (Figure 1). This confirms the previous report by Farber et al. (1971). In the experiment of Figure 1, about 54% of the cells were stimulated to proliferate, reaching a maximum at 28.5 hr, while the peak of mitoses occurred between 26 and 30 hr (not shown).

However, when confluent WI-38 cells were trypsinized and replated at a lower density (2×10^4 cells/cm^2) in fresh medium containing 10% fetal calf serum, the wave of DNA synthesis began only at the 18th hr after replating. The percentage of cells stimulated to proliferate was 57% at 32 hr, while mitoses reached a peak between 30 and 32 hr. In unstimulated cultures, the percentage of cells labeled by [^{3}H]thymidine over a period of 24 hr was 2.4%.

These data show that there is a lengthening of the "prereplicative" phase of approximately 5 hr in trypsinized cells seeded at a lower density with respect to serum-stimulated cultures.

Nuclear Protein/DNA Ratio of Isolated Nuclei. When confluent monolayers were stimulated to proliferate by serum, there were no obvious changes in the protein/DNA ratio of nuclei throughout the different time intervals after stimulation (Table I). However, within 1 min after trypsin treatment of confluent monolayers, there was a 40–50% loss of nuclear protein. This decrease in the nuclear protein/DNA ratio persisted until 6–8 hr after replating (Table I). Thus, by 6–8 hr after replating the amount of nuclear proteins in trypsinized cells has simply returned to the level of confluent nontrypsinized WI-38 cells.

RNA Synthesis in Isolated Nuclei of WI-38 Cells. The kinetics of incorporation of nucleoside triphosphates into RNA were essentially identical for nuclei isolated from 8th day confluent cultures, from serum-stimulated cells, and from trypsinized cells. The conditions of assay were those described by Marzluff et al. (1973). As shown in Figure 2, the rate of incorporation of [^{3}H]UTP into RNA was linear for 15 min and leveled off after 20 min. For confluent monolayer nuclei, the rate was approximately 2 pmol of [^{3}H]UTP/μg of DNA over 15 min (Figure 2). Although the nuclei isolated this way were not absolutely free of cytoplasmic contamination (DNA/RNA/protein, 1:1:6), the incorporation of [^{3}H]UTP was negligible in the absence of the other three nucleoside triphosphates (Figure 2). This indicated there was no appreciable precursor pool of nucleoside triphosphates in these preparations. As shown in Figure 3, the rate of incorporation was linearly dependent on the concentration of nuclei.

RNA Synthesis after Serum Stimulation. Confluent monolayers were stimulated by changing the medium as described under Materials and Methods. RNA synthesis was determined in nuclei isolated at various times after the nutritional change. As mentioned above, incorporation of [^{3}H]UTP was linear up to 15 min and only this value was taken into account in determining the points in Figure 4. Each point on Figure 4 represents an average of at least three separate assays from different sets of experiments with a standard deviation of approximately 5%.

RNA synthesis in nuclei was found to increase above control level as early as 30 min after the change of medium. Within 2 to 3 hr, there was an overall increase of 40% (see also Table II). The incorporation of [^{3}H]UTP remained constant from 4 to 6 hr after stimulation. At 8 hr there was a further increase up to 100% above control nuclei. Thereafter the increase slowly leveled off (Figure 4), the 17th hr point being the last to be determined.

Conditioned medium or fresh medium gave the same results provided 10% fetal calf serum was added to the medium. When cells were placed in 0.3% serum in fresh medium for 3 hr, the [^{3}H]UTP incorporation into RNA by isolated nuclei was not increased above controls (see Table II). This concentration of serum does not cause stimulation of cell proliferation in resting confluent monolayers of WI-38 cells (Rovera and Baserga, 1973).

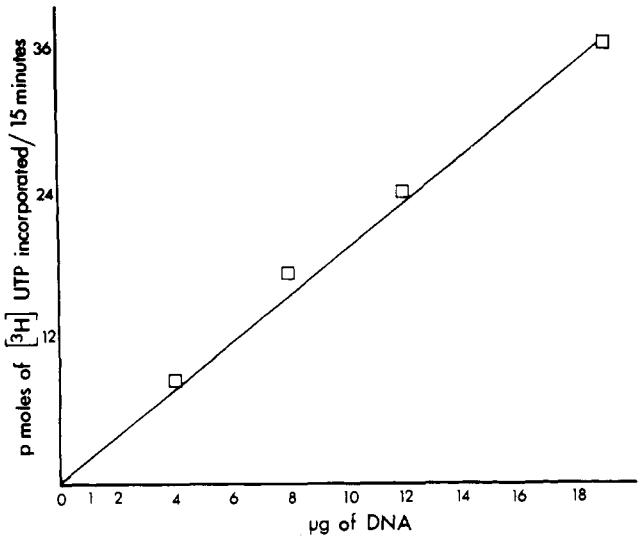

FIGURE 3: Effect of nuclei concentration on [^{3}H]UTP incorporation into RNA. Aliquots with different nuclei concentrations were incubated for 15 min at 37° as described in Figure 2.

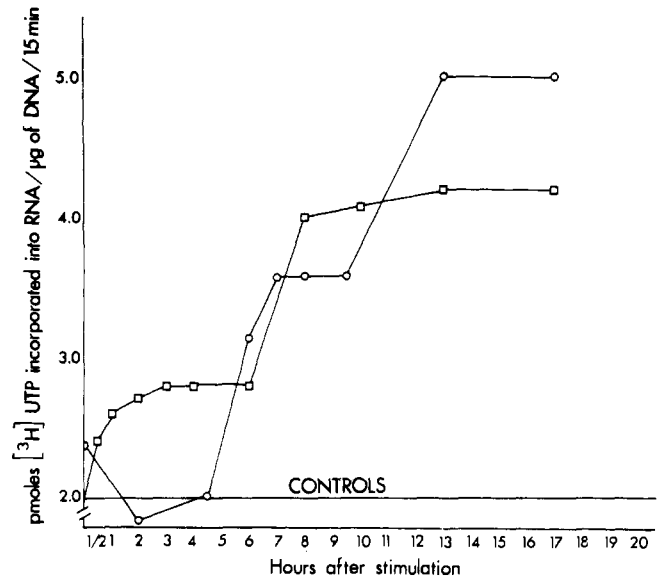

FIGURE 4: RNA synthesis in isolated nuclei of WI-38 cells at different times after nutritional change or trypsinization and replating at a lower density. Nuclei from confluent monolayers were used as control: (□) nuclei from cells stimulated by 10% fetal calf serum; (○) nuclei from cells trypsinized and replated at lower density. Incorporation of [^{3}H]UTP was determined as in Figure 2. The values given are those after 15 min of incubation.

RNA Synthesis in Nuclei of Cells after Trypsinization and Replating. Confluent monolayers of cells were trypsinized and replated at a lower density in fresh medium containing 10% fetal calf serum. RNA synthesis was studied in these nuclei, at various time intervals after replating, as described above.

Immediately after the cells were trypsinized, there was a transient increase of 10–20% in [^{3}H]UTP incorporation. It dropped back to control levels at 2 hr and began to increase again between the 5th and 7th hr to 80% above control (Figure 4). From the 7th to the 9th hr, the incorporation remained constant. At the 13th hr there was another increase which reached a level of about 170% above controls. Thereafter the increase remained at the same level until the 17th hr (Figure 4).

To briefly summarize up to this point, it appears that changes in RNA synthesis are very similar in cells stimulated in either way. However, there is an apparent delay of 6 hr in the increase in RNA synthesis in trypsinized and replated cells. There is a biphasic increase between 0–2 and 6–8 hr in serum-stimulated cells, and between 5–7 and 10–12 hr in trypsinized cells replated at lower density.

The transient increase of [^{3}H]UTP incorporation observed in confluent monolayers immediately after trypsinization could not be reproduced in a confluent culture which had just been stimulated with fresh medium plus 10% fetal calf serum for 3 hr (Table III). On the contrary, there was a 20% decrease of template activity in nuclei of cells stimulated for 3 hr after 1-min trypsin treatment. In other words, the difference in [^{3}H]UTP incorporation between the resting confluent monolayers and serum-stimulated cells can be abolished if the cells are harvested with trypsin (Table III). Chymotrypsin produced the same effect (Table III).

Circular Dichroism of Nuclei. Figure 5 shows the circular dichroism spectra of nuclei obtained from cells 3 hr after serum stimulation or 6 hr after trypsinization and replating at lower density. Nuclei from unstimulated confluent monolayers were used as controls.

There are definite increases in maximum positive ellipticity in the 250–300-nm region of CD spectra of nuclei from cells stimulated in either way in comparison to controls. The $[\theta]_{max}$ around 275 nm for controls, cells 3 hr after serum stimulation, and cells 6 hr after trypsinization and replating are, respectively, 3400, 4550, and 4770 deg cm^2/dmol of nucleotide residue or a ratio of 1:1.33:1.40. The value for control nuclei is similar to the one reported for rat liver nuclei by Wagner and Spelsberg (1971).

Table II: Incorporation of [^{3}H]UTP into RNA by Isolated Nuclei of WI-38 Cells.[a]

Nuclei from	[^{3}H]UTP Incorpn. by Isolated Nuclei (pmol/μg of DNA per 15 min)	% of Incorpn. above Control
Controls	1.90	0
3 hr after addition of 10% serum to old medium	2.75	45
3 hr after replacement of old medium with fresh medium plus 10% serum	2.71	43
3 hr after replacement of old medium with fresh medium plus 0.3% serum	1.99	4.7
6 hr after trypsinization and replating in fresh medium with 10% serum	3.06	61.1
6 hr after trypsinization and replating in fresh medium with 0.3% serum	2.16	13.7

[a] Nuclei were isolated at different time intervals after changing medium with or without prior trypsin treatment. Conditions for [^{3}H]UTP incorporation into these nuclei are described under Materials and Methods. Nuclei from resting confluent monolayers were used as controls.

Circular Dichroism Spectra of Ethidium Bromide–Nuclei Complexes. Figure 6 shows the circular dichroism spectra of ethidium bromide bound to nuclei from cells 3 hr after serum stimulation or 6 hr after trypsinization and replating at lower density. As in the last section, nuclei from cells in a resting confluent monolayer were used as control.

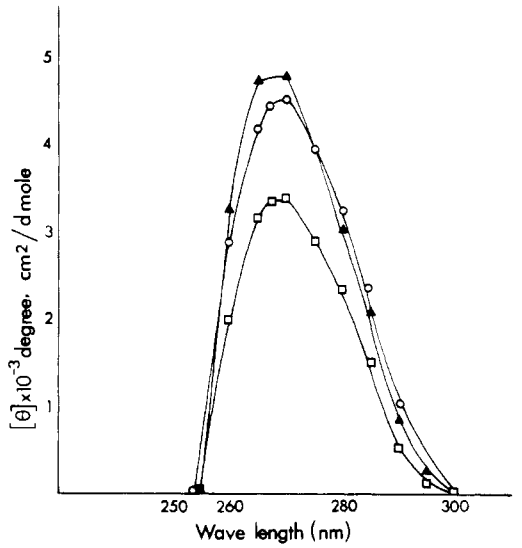

FIGURE 5: Circular dichroism spectra of isolated WI-38 nuclei: (□) nuclei from resting confluent monolayers; (○) nuclei from confluent monolayers of cells stimulated for 3 hr with 10% fetal calf serum in fresh medium; (▲) nuclei from confluent cells trypsinized and replated at lower density for 6 hr in 10% fetal calf serum with fresh medium. Measurements were made on nuclei in 0.01 *M* Tris-HCl (pH 8.0). The rest of the experimental details are given under Materials and Methods. Results are expressed in terms of mean ellipticity [θ] where the dimensional units are in deg cm^2 per dmol of nucleotide residue.

Table III: Effect of Trypsin and Chymotrypsin on RNA Synthesis in Isolated Nuclei.[a]

Enzymatic Treatment	[^{3}H]UTP Incorpn. by Isolated Nuclei (pmol/μg of DNA per 15 min)	
	Controls	Serum-Stimulated
None	1.90	2.71
With trypsin	2.26	2.33
With chymotrypsin	2.69	2.71

[a] Confluent monolayers of WI-38 cells were stimulated with 10% fetal calf serum in fresh medium for 3 hr. The cells were then harvested with or without 1-min pretreatment of either 0.25% trypsin or 0.1% chymotrypsin at room temperature. The nuclei were isolated and [^{3}H]UTP incorporation studies carried out as described. Unstimulated confluent monolayers were used as controls. The experiments were repeated at least three times. The figures presented are averages with a standard deviation of about 5%.

At the optimum dye/DNA-P ratio of 0.25, the $[\theta]_{308}$ of the ethidium bromide–nuclei complex for control cells 3 hr after serum stimulation and cells 6 hr after trypsinization and replating are, respectively, 22,800, 28,500, and 29,820 deg cm^2/dmol of dye, or a ratio of 1:1.25:1.31.

The experiments on circular dichroism and ethidium bromide binding have been repeated twice. Although there were some slight fluctuations in absolute values, the differences between control and stimulated cells were essentially similar in the three experiments.

Discussion

In this study nuclei were isolated from WI-38 cells for direct functional and structural studies. Isolated nuclei in this system are capable of synthesizing RNA at a reasonable rate. Although this nuclear preparation is not absolutely free of cytoplasmic contamination, it is free of any nucleotide triphosphate precursor pool which can greatly alter the

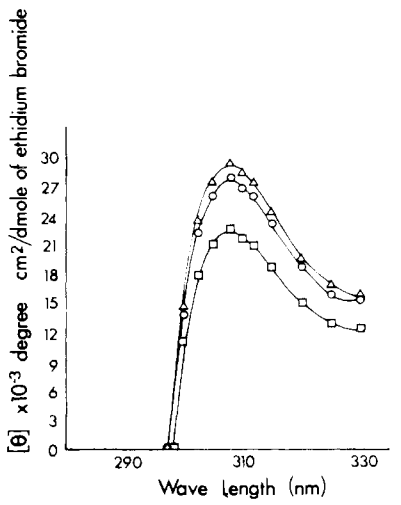

FIGURE 6: Circular dichroism spectra of ethidium bromide bound to isolated WI-38 nuclei: (□) confluent monolayers of WI-38 cells; (○) confluent monolayers stimulated for 3 hr with 10% fetal calf serum in fresh medium; (△) confluent monolayers of cells trypsinized and replated for 6 hr in 10% fetal calf serum with fresh medium. DNA concentrations were determined by diphenylamine. Dye and DNA-P ratios were kept at optimum *r*, 0.25.

measurement of RNA synthesis in whole cells (Todaro et al., 1965; Cunningham and Pardee, 1969). RNA products of isolated nuclei have not been analyzed. However, using similar methods in the mouse myeloma cell system, Marzluff et al. (1973) were able to show the synthesis of RNA species of different molecular weights including species with poly(A) stretches.

Confluent monolayers of WI-38 can be stimulated to divide either by serum or by trypsinization and replating at lower density. Our data on the template activity of nuclei show that within 30 min after serum stimulation RNA synthesis increases. In the first 12 hr after stimulation and before the initiation of DNA synthesis, there is a biphasic incresase in template activity.

An increase in RNA synthesis in nuclei of cells that were trypsinized and replated is not detectable until 6 hr after the treatment. Thereafter, there is also a biphasic increase in RNA synthesis before the onset of DNA synthesis (Figure 4).

To correlate these changes in template activity structural studies were also carried out on nuclei from cells either 3 hr after serum stimulation or 6 hr after trypsinization and replating.

Circular dichroism has been extensively used to determine the structure of chromatin (see review by Simpson, 1973). In many cases the increase in positive ellipticity of chromatin in the 250–300-nm region has been related to an increase in transcriptional activity (Polacow and Simpson, 1973; Spelsberg et al., 1973; Baserga, 1974). Structural changes related to function in general have also been reported in chromatin and/or protein–DNA complexes by several authors (Kleiman and Huang, 1971; see review by von Hippel and McGhee, 1972). In the present study, conformational changes did occur in nuclei with higher template activity whether from cells stimulated by serum or from cells trypsinized and replated. The increase in maximum ellipticity over the 250–300-nm region (Figure 5) suggests a structural change accompanying the increase in transcriptional activity of the nuclei. The differences observed in CD spectra and ethidium bromide binding be-

tween control and stimulated cells are of such magnitude that they cannot be explained by variations in light scattering (Wagner and Spelsberg, 1971; Olins and Olins, 1972) or differences in the amount of RNA per nucleus, which does not vary appreciably in WI-38 cells in the first 6 hr after stimulation (Farber et al., 1971). Of course, qualitative changes in RNA cannot be ruled out (see above).

It was suggested by Dalgleish et al. (1971) that the spectropolarimetric method gives the number of primary ethidium bromide binding sites on DNA and that the outside or weakly bound dye molecules do not significantly contribute to the observed optical activity. This study has been confirmed and further extended on chromatin by Williams et al. (1972) and on chromatin of stimulated WI-38 cells by Nicolini and Baserga (1975). A linear relationship between binding of ethidium bromide and template activity of isolated avian chromatin has been reported by Seligy and Lurquin (1973). A number of investigators have also reported an increased binding of intercalating dyes, such as Acridine Orange (Darzynkiewicz et al., 1969; Zetterberg and Auer, 1970; Smets, 1973; Alvarez, 1974) and ethidium bromide (Ringertz and Bolund, 1969), by nuclei of cells stimulated to proliferate. These studies, although carried out on whole cells, are in agreement with our present observation in isolated nuclei. As shown in Figure 6, the primary binding sites of nuclei from cells stimulated to proliferate were obviously increased above controls. Moreover, there is a correlation between the binding of the dye and the increase in positive ellipticity in CD spectra of nuclei (compare Figures 5 and 6).

Our results, therefore, can be summarized as indicating three findings of interest, namely: (1) functional and structural changes occur in nuclei of cells stimulated to proliferate by trypsinization and replating at lower density; (2) the changes, like the onset of DNA synthesis, are delayed by 5 hr in respect to serum-stimulated cells; and (3) the changes described in nuclei of serum-stimulated WI-38 cells mirror reasonably well those already reported in isolated chromatin from the same cells.

It remains to consider an explanation for the delay occurring in cells trypsinized and replated at a lower density.

One possibility is that trypsin may act directly on nuclear proteins and, indeed, Simpson (1972) has shown that direct limited tryptic digestion of native chromatin causes it to change its CD spectra toward the direction of protein-free DNA. However, apart from the consideration that no evidence has yet been brought forward that trypsin can enter the nuclei of intact cells, our data show that the template activity of isolated nuclei, after a brief, modest increase immediately after trypsinization, returns to control level until the 6th hr. Although trypsin may be directly responsible for the ephemeral early increase, another explanation must be sought for the delay in template activity increase occurring in cells trypsinized and replated at a lower density.

After confluent cells are trypsinized, there is a loss of 40–50% of nuclear proteins (Table I), most of which are nonhistone chromosomal proteins (Maizel et al., 1975). Only between 6 and 10 hr after replating does the amount of nonhistone proteins return to the level of confluent monolayers. It is tempting to suggest that the nonhistone chromosomal proteins which are lost from nuclei by trypsinization are necessary for the cells to enter the prereplicative phase, although other possible factors cannot be ruled out, such as the possible loss of membrane or membrane associated proteins during trypsinization. The analysis of these proteins is currently in progress.

For the moment, it can be concluded that stimulation of cell proliferation by trypsinization and replating at lower density offers a valuable model (especially when compared to serum-stimulated monolayers) for investigating the relationship of nuclear proteins to template activity and cell proliferation.

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A Possible Alternate Pathway of Bacteriochlorophyll Biosynthesis in a Mutant of *Rhodopseudomonas sphaeroides*†

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ABSTRACT: A previously uncharacterized bacteriochlorophyll-less mutant (mutant 8) of *Rhodopseudomonas sphaeroides* has been found to excrete a tetrapyrrole–protein complex into the incubation medium. The structure of the major pigment of the complex was characterized as 2-desacetyl-2-vinylbacteriopheophorbide. The corresponding magnesium derivative does not fit into the currently proposed biosynthetic pathway for bacteriochlorophyll, and thus may indicate the existence of an alternate pathway of bacteriochlorophyll synthesis in this organism. Such an alternate pathway would be possible if reduction from the chlorin to the tetrahydroporphyrin stage can occur either before or after hydration of the 2-vinyl substituent of chlorophyllide *a* to an α-hydroxyethyl group.

A plausible sequence for the latter stages (or magnesium branch) of Bchl[1] synthesis is shown in Figure 1. This pathway was proposed on the basis of work carried out with mutants of *Rhodopseudomonas sphaeroides* unable to synthesize either Bchl (Griffiths, 1962; Lascelles, 1966; Lascelles and Altshuler, 1967; Richards and Lascelles, 1969) or carotenoids (Sistrom et al., 1956; Stanier and Smith, 1959), and with the wild-type strain inhibited by 8-hydroxyquinoline (Jones, 1963a,b, 1964, 1967). In all of these studies, tetrapyrrole pigments were excreted into the growth medium. After isolation and identification, the compounds were arranged in a logical sequence which would accomplish the synthesis of Bchl (Figure 1). In all cases, the true intermediates were assumed to be the magnesium chelates, whether they or the corresponding magnesium-free derivatives were actually isolated. Lascelles (1966) observed that the pigments were actually excreted as lipoprotein-bound complexes, and suggested that these might represent a natural "carrier protein" complex required for Bchl synthesis. No intermediate (assumed to be magnesium 2-vinylpheoporphyrin a_5) between P-631 and P-665 or any intermediates involved in the cyclization of the cyclopentenone ring have been identified in *R. sphaeroides* (Figure 1). The addition of phytol was assumed to be the final step in Bchl formation, since none of the intermediates contained a phytyl ester. The only enzyme of the magnesium branch to be demonstrated in cell-free extracts has been magnesium-protoporphyrin:*S*-adenosylmethionine methyltransferase (EC 2.1.1.11) by Gibson et al. (1963). Lascelles and Hatch (1972) have pointed out, however, that the mutants should be good sources of intermediates for future enzymatic studies.

In studies with closely related strains of *Athiorhodaceae*, Krasnovskii et al. (1970) have detected compounds spectroscopically similar to magnesium 2,4-divinylpheoporphyrin a_5, chlorophyll *a*, and 2-devinyl-2-α-hydroxyethylchlorophyll *a* in mutants of *Rhodopseudomonas palustris*. The pigments were found to be mixtures of the phytyl esters and the corresponding nonphytylated derivatives. Drews et al. (1971) have isolated a pigment–protein complex from a mutant of *Rhodopseudomonas capsulata* containing the phytyl esters of both magnesium 2-vinylpheoporphyrin a_5 and the corresponding magnesium-free derivative. Oelze and Drews (1970) have also isolated a pigment–protein complex from a mutant of *Rhodospirillum rubrum*. However, in this case the pigments excreted in the complex were the nonesterified pheophorbide *a* and 2-devinyl-2-α-hydroxyethylpheophorbide *a*. Schick and Drews (1969) had earlier described another mutant of *R. rubrum* which excreted a much different pigment–protein complex containing bacter-

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[1] Abbreviations used are: Bchl, bacteriochlorophyll; DDBQ, 2,3-dichloro-5,6-dicyanobenzoquinone.