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Gene Activation in WI-38 Fibroblasts Stimulated to Proliferate: Requirement for Protein Synthesis

(template activation/human diploid fibroblasts/cell proliferation)

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ABSTRACT **Confluent monolayers of WI-38 human diploid fibroblasts can be stimulated to divide by fresh medium containing 30% fetal-calf serum. Up to 80% of the cells are stimulated to divide, with a peak of DNA synthesis between 15 and 21 hr. 1 hr after the change of medium there is a 70% rise in chromatin template activity. Cycloheximide inhibited the increase in chromatin template activity. A requirement for RNA synthesis was investigated by incubating stimulated and unstimulated cells with 10 μg/ml of actinomycin D. In spite of a 95% inhibition of RNA synthesis in whole cells, purified chromatin from stimulated cells showed the usual increase in template activity. These experiments implicate a requirement for protein synthesis in template activation, and imply that the synthesis of this (or these) protein(s) is independent of RNA synthesis and regulated by a purely translational mechanism.**

When human diploid fibroblasts in cell culture form confluent monolayers, DNA synthesis and cell division almost completely cease (1–4), and more than 99% of the cells can be considered to be in a quiescent state. If the medium is changed or new serum is added, these density-inhibited cultures can be stimulated to proliferate again, with a lag period of about 12–15 hr between the application of the stimulus and the onset of DNA synthesis (2–5). Several biochemical changes have been described during the prereplicative phase preceding the onset of DNA synthesis, namely: a rapid decrease in the intracellular pool size of most amino acids, which occurs in the first 2–3 hr after stimulation (3); an increase in the uptake of [^{3}H]uridine and its incorporation into RNA (3, 5); an increased synthesis of acidic nuclear proteins, which occurs as early as the first hour after stimulation (4); and an increase in template activity of isolated chromatin, which begins as early as 1 hr after stimulation, remains elevated for another 10 hr, and increases further at 12 hr (5). In the last-mentioned experiment, template activity was measured in an *in vitro* system that used chromatin isolated from either stimulated or unstimulated cells and exogenous *Escherichia coli* RNA polymerase. The results were interpreted as indicating that, shortly after the cells are stimulated to proliferate, there is activation of the genome (5).

Genome activation as one of the early events in the prereplicative phase of stimulated DNA synthesis and cell division has been shown directly or indirectly in several systems (5–10), but the mechanisms regulating such activation have not been identified. It has been suggested that gene function in mammalian cells may be regulated by proteins, either through *de novo* synthesis or structural modifications (11–15) or by low-molecular-weight nuclear RNA (16, 17). Each proposed mechanism does not exclude the other if we suppose that different levels of gene regulation, involving permanent or temporary modification of gene function, may exist (18). *De novo* synthesis of acidic nuclear proteins has been recently proposed as the mechanism for gene activation in some G_0 cells stimulated to synthesize DNA and divide, specifically, the estrogen-stimulated uterine cells (19), the isoproterenol-stimulated salivary gland cells (20), and the WI-38 human diploid fibroblasts stimulated by a change of medium (4).

In the present communication we show that protein synthesis is required for the increase in template activity of chromatin occurring 1 hr after WI-38 human diploid fibroblasts are stimulated to proliferate by a change of medium, and that this increase does not require RNA synthesis.

MATERIALS AND METHODS

Cell culture

WI-38 human diploid fibroblasts, purchased from Flow Laboratories (Rockville, Md.), were grown as described previously (3, 4) in 1-liter Blake bottles. Confluent monolayers of cells, usually on the seventh day after plating and with fewer than 1% of the cells in DNA synthesis, were stimulated to proliferate by replacing the old medium with fresh medium containing 30% fetal-calf serum (5). Under these conditions up to 80% of the contact-inhibited WI-38 cells can be stimulated to synthesize DNA and divide. In some experiments the cells were "pulse-stimulated" for 3 hr only, that is, the conditioned medium was replaced with fresh medium containing 30% fetal calf serum for 3 hr and then the fresh medium was, in turn, replaced by conditioned medium supplemented with 2 mM L-glutamine. Under these conditions of "pulse stimulation" some 20–30% of contact-inhibited WI-38 fibroblasts are stimulated to proliferate.

Template activity of the chromatin

The chromatin was isolated, with minor modifications, by the method of Marushige and Bonner (21) as described elsewhere (5). RNA polymerase was prepared from early-log-phase *E. coli* strain B (General Biochemicals, Chagrin Falls, Ohio) (22). The complete incubation mixture for chromatin template activity for RNA synthesis contained, in a final volume of 250 μl: 10 μmol of Tris buffer, pH 8.0; 1 μmol of $MgCl_2$; 0.25 μmol of $MnCl_2$; 3 μmol of β-mercaptoethanol; 0.1 μmol of CTP, GTP and UTP; 0.1 μmol of [^{14}C]ATP (1 Ci/mol); variable amounts of chromatin; and 18 μg of F_4 *E. coli* polymerase. Incubation was carried out at 37°C for 10 min and terminated by addition of ice-cold 10% tri-

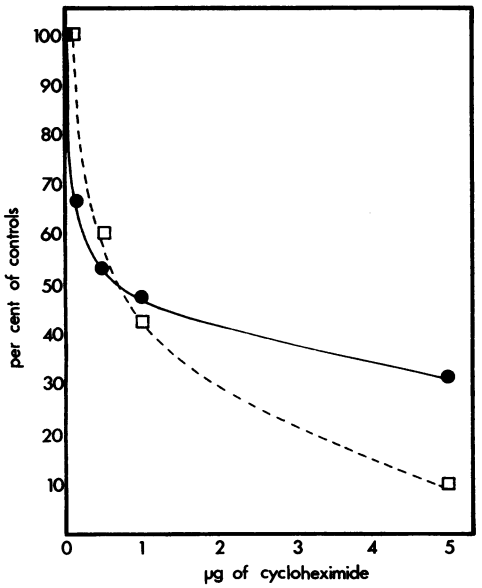

FIG. 1. Effect of cycloheximide on protein synthesis (●—●) and on stimulated DNA synthesis (□——□). Confluent monolayers of human diploid fibroblasts were stimulated with fresh basal Eagle's medium containing 30% fetal-bovine serum in the presence of increasing concentrations of cycloheximide. 1 hr later the cells were incubated with 1 μCi/ml of [^{3}H]leucine for 30 min. For the determination of DNA synthesis, cells were stimulated in the presence of cycloheximide for 3 hr, after which time the cells were washed with warm medium and further incubated in exhausted medium supplemented with 2 mM glutamine. 12 hr after stimulation, 1 μCi/ml of [^{3}H]thymidine was added. The untreated controls had a specific activity for proteins of 184 (±6) cpm/μg of proteins and for DNA of 5780 (±274) cpm/μg of DNA.

chloroacetic acid (5). The acid-insoluble material collected on millipore filters was dissolved in 1 ml of Cellosolve and counted in 15 ml of Cellosolve–toluene scintillation mixture (23) at 80% efficiency.

Other biochemical determinations

Protein synthesis was assayed by determining the incorporation of [^{3}H]leucine into acid-precipitable material. The cells were incubated in Hank's balanced salt solution containing 1 μCi/ml of [^{3}H]leucine as described previously (4). Protein amounts were determined by the method of Lowry *et al.* (24). To study DNA synthesis, we added 1μCi/ml of [^{3}H]thymidine 12 hr after stimulation of cell proliferation. The experiments were terminated at 24 hr and the amount of radioactivity incorporated into DNA was determined. DNA amounts were obtained by the diphenylamine method (25). RNA synthesis was investigated by incubating cells for 30 min in the presence of 1 μCi/ml of [5-^{3}H]uridine. The specific activity of RNA was determined by the method of Scott *et al.* (26).

Radioactivity in the various fractions was determined by counting 1 ml in a Triton–toluene mixture (27). Chromatin proteins were fractionated in a histone fraction and in three acidic fractions (soluble in 0.15 M NaCl, soluble in 0.35 M NaCl, and insoluble in acid), as described by Stein and Baserga (28).

Chemicals

Cycloheximide (Actidione) was purchased from Nutritional Biochemicals, and actinomycin D from Mann Research Laboratories. [*Methyl*-^{3}H]thymidine (14.1 Ci/mmol) was purchased from Amersham Searle, Arlington Heights, Ill. [5-^{3}H]uridine (25.9 Ci/mmol), [^{14}C]ATP (54 Ci/mol) and [^{3}H]leucine (55.2 Ci/mmol) were purchased from New England Nuclear Corp. and [^{3}H]actinomycin D (3.38 Ci/mmol) from Schwarz BioResearch. CTP, GTP, and UTP were from Calbiochem. All other chemicals were of reagent grade.

TABLE 1. *Proliferative capacity of human diploid fibroblasts after 3-hr treatment with cycloheximide*

Treatment	cpm/μg DNA
Unstimulated cells	34 (±6)
Control cells stimulated for 3 hr and restimulated for 21 hr	1580 (±165)
Cells stimulated in the presence of cycloheximide for 3 hr and restimulated for 21 hr	1660 (±140)

Confluent WI-38 cells were stimulated to proliferate by the addition of fresh medium containing 30% fetal-calf serum for 3 hr, with or without cycloheximide (5 μg/ml). The cells were incubated for another 3 hr in exhausted medium and then stimulated again with fresh medium containing 10% serum. 21 hr after the second stimulation, the cells were incubated for 1 hr in the presence of 1 μCi/ml of [^{3}H]thymidine.

RESULTS

Effect of cycloheximide on stimulation of DNA synthesis

To study the relationship between protein synthesis and the increased template activity of chromatin isolated from stimulated cells, we used cycloheximide to inhibit protein synthesis. The dose of cycloheximide to be used was chosen on the basis of the experiments shown in Fig. 1 and Table 1. Fig. 1 shows the effect of increasing concentrations of cycloheximide on the incorporation of [^{3}H] leucine into acid-precipitable material in cells incubated for 1 hr with cycloheximide prior to a 30-min exposure to [^{3}H]leucine. A concentration of 5 μg of cycloheximide per ml causes in these cells a 65% inhibition of protein synthesis. Fig. 1 also shows the effect that the same concentrations of cycloheximide have on stimulation of DNA synthesis. For these experiments, cycloheximide was added to the cells for 3 hr, during a pulse stimulation of confluent monolayers with fresh medium containing 30% serum. Cycloheximide and stimulating medium were then removed and replaced with conditioned medium and the stimulation of DNA synthesis was measured as described in Fig. 1. A concentration of 5 μg/ml of cycloheximide added during the pulse stimulation causes a 90% inhibition of the stimulation of DNA synthesis. It may be objected that these concentrations of cycloheximide are toxic and cause cell death. Table 1 shows that a concentration of 5 μg/ml of cycloheximide does not cause cell death or marked damage in WI-38 fibroblasts. When cells were exposed to cycloheximide for 3 hr and then stimulated again by the addition of fresh medium, they responded with DNA synthesis as vigorously as stimulated cells not exposed to cycloheximide. These results indicated that the viability of WI-38 cells was not impaired by a 3-hr exposure to cycloheximide and that the induction of DNA synthesis is dependent upon proteins synthesized in the first hour after the application of the stimulus, in accordance with previous results from this laboratory (4).

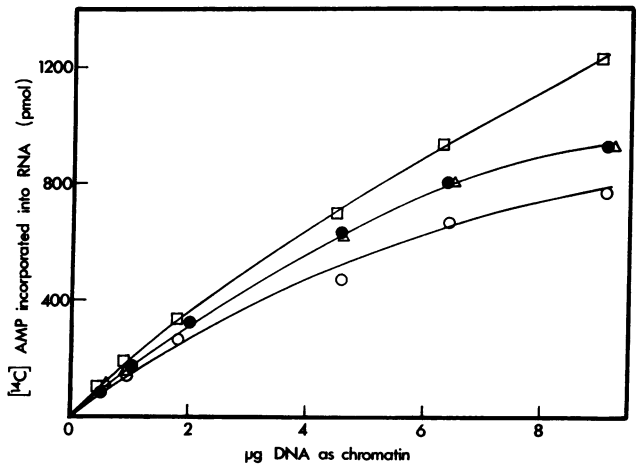

FIG. 2. Template activity of chromatin isolated from unstimulated cells and from cells 1 hr after stimulation by a change of medium. Unstimulated cells (O—O). Unstimulated cells treated for 1 hr with 5 μg/ml of cycloheximide (Δ—Δ). Cells stimulated for 1 hr (□—□). Cells stimulated for 1 hr in the presence of cycloheximide (●—●).

Effect of cycloheximide on template activity of chromatin

Fig. 2 shows the effect of cycloheximide (5 μg/ml) on template activity of chromatin. As described in a previous paper (5), stimulation of WI-38 cells by change of medium containing 30% serum causes an increase in template activity of chromatin. Cycloheximide inhibits the increase in template activity of chromatin isolated from stimulated cells, which indicates a requirement for protein synthesis for the activation of the genome. The same concentration of cycloheximide causes a modest stimulation of chromatin template activity (Fig. 2) in confluent nonproliferating monolayers of WI-38 cells. The template activity of cycloheximide-treated stimulated and unstimulated cells was thus identical, and higher than in unstimulated cells not exposed to cycloheximide. This raises the question whether cycloheximide *per se* could increase gene function through inhibition of protein synthesis.

Fig. 3 shows the effect of cycloheximide on the incorporation of [^{3}H]uridine into RNA of stimulated WI-38 cells. As previously reported (3, 5), a change of medium causes a rapid increase in RNA synthesis in WI-38 cells. This increase is largely inhibited when the stimulation is done in the presence of a concentration of 5 μg/ml of cycloheximide, although the values for cycloheximide-treated stimulated cells are still above the control values of unstimulated cells. A concentration of 0.1 μg/ml of cycloheximide, a dose that inhibits neither stimulation of DNA synthesis nor cell division, causes an increase in the incorporation of [^{3}H]uridine into RNA 20% above the values of stimulated cells.

Effect of cycloheximide on the synthesis of nonhistone chromosomal proteins

Recent experiments have indicated that nuclear acidic proteins may be involved in the regulation of gene activation that occurs when cells are stimulated to synthesize DNA and divide (4, 19, 20, 28). Table 2 shows that after the stimulation of cell proliferation by change of medium there is an increased synthesis of acidic chromosomal proteins in diploid fibroblasts and that cycloheximide, at a concentration of 5 μg/ml, almost completely inhibits the synthesis of these proteins. This observation, of course, does not demonstrate that acidic nuclear proteins are involved in the regulation of gene function, but is compatible with that hypothesis.

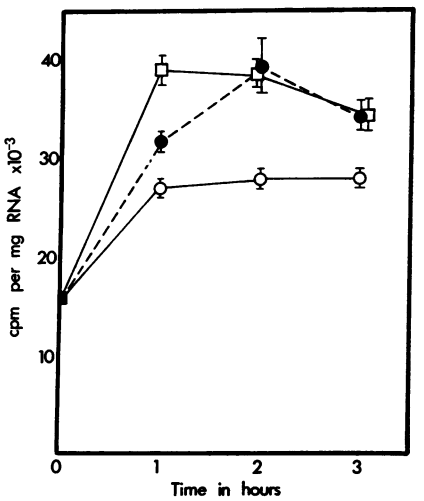

FIG. 3. Effect of cycloheximide on the incorporation of [^{3}H]uridine into RNA of stimulated WI-38 cells. Cells were stimulated by changing to fresh medium containing 30% fetal-bovine serum. At the times after stimulation indicated on the abscissa, the cells were incubated for 30 min with 1 μCi/ml of [5-^{3}H]uridine and the specific activity of RNA was determined by the method of Scott *et al.* (26). Stimulated cells not exposed to cycloheximide (●—●). Cells stimulated in the presence of 0.1 μg/ml (□—□) or of 5 μg/ml (O—O) of cyclohexmimde.

Effect of actinomycin D on template activity of chromatin

The results thus far indicate that protein synthesis is required for the increase in chromatin template activity occurring in the first hour after stimulation of WI-38 fibroblasts. The question arose whether previous RNA synthesis was also necessary for the increase in template activity. To test this possibility, we treated unstimulated cells, and cells stimulated to proliferate, with actinomycin D (10 μg/ml) for 1 hr. This dose inhibits more than 90% of RNA synthesis in WI-38 cells within 3 min after administration of the drug. As shown in

TABLE 2. *Effect of cycloheximide on the synthesis of chromatin proteins*

	Specific activity of chromatin fractions (cpm/μg proteins)		
	Soluble in 0.15 M NaCl	Soluble in 0.35 M NaCl	Residual proteins
Unstimulated cells	39.5	87	74.1
Unstimulated cells treated for 1 hr with cycloheximide	3.3	3.1	10.1
Cells stimulated for 1 hr	67.9	164	119.7
Cells stimulated for 1 hr in the presence of 5 μg/ml cycloheximide	5.1	7.6	7.7

Confluent cells and cells stimulated to proliferate for 1 hr with fresh medium containing 30% serum, with or without cycloheximide (5 μg/ml), were labeled for 30 min with 0.5 μCi/ml of [^{3}H]leucine.

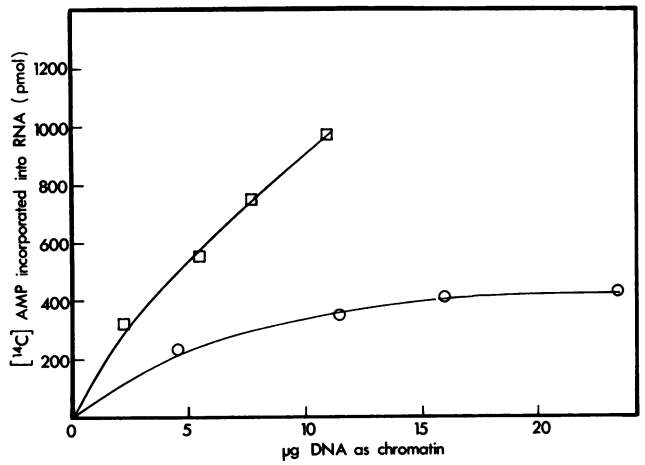

FIG. 4. Effect of actinomycin D on template activity of chromatin of non-proliferating cells (O—O) and of cells stimulated to proliferate for 1 hr (□—□). WI-38 cells in confluent monolayers or stimulated to proliferate by 30% fetal-bovine serum were treated for 1 hr with 10 μg/ml of actinomycin D. Template activity was assayed after 18 hr of dialysis against 2000 vol of 0.01 M Tris buffer, pH 8.0, as detailed in ref. 5.

Fig. 4, the increase in template activity of chromatin from stimulated cells is not inhibited when the stimulation occurs in the presence of 10 μg/ml of actinomycin D. It should be noted that actinomycin D is partially removed from DNA during the preparation of chromatin and its subsequent dialysis. Thus, in these experiments, gene function was suppressed during stimulation but was partially restored during the preparation of chromatin. To determine the amount of actinomycin D lost during chromatin preparation, we labeled cells beforehand with 1 μCi/ml of [^{3}H]actinomycin D. In chromatin preparations of both stimulated and unstimulated cells, the amount of actinomycin D bound per mg of DNA was less than 40% of the amount originally bound to nuclei (also expressed per mg of DNA).

DISCUSSION

Previous experiments by Chaudhuri and Lieberman (29) and by Neal *et al.* (30) indicated that cycloheximide could inhibit the increase in RNA synthesis that occurs in regenerating liver after partial hepatectomy. These authors, however, investigated only the effect of cycloheximide on RNA synthesis some time after partial hepatectomy. Neal *et al.* (30) have also shown that cycloheximide added to the incubation mixture does not affect the template activity of isolated nuclei.

Our observations indicate that one of the very early events following the stimulation of WI-38 cells to proliferate, namely, the increase in chromatin template activity occurring at 1 hr (5), is dependent on the synthesis of proteins, possibly acidic nuclear proteins. In addition, our experiments seem to indicate that other genes, not necessarily connected with cellular proliferation, are usually in a repressed state because of continuous protein synthesis. Inhibition of protein synthesis by cycloheximide may cause the activation of some segments of the genome. An increased incorporation of uridine into RNA after cycloheximide has also been observed *in vivo* in the intestinal epithelium of the rat (E. Farber, personal communication). The results indicate, then, that activation of the genome may follow two different and opposite mechanisms: in some cases, as for instance the activation of the genome that leads to DNA synthesis and cell division, it may require the synthesis of special proteins, while in other cases it requires the inhibition of the synthesis of proteins that ordinarily repress the genome.

The experiments with elevated doses of actinomycin D show that an almost complete inhibition of RNA synthesis does not affect the increase in template activity of chromatin 1 hr after stimulation of cell proliferation. This same phenomenon has been observed *in vivo* in kidneys of mice stimulated to synthesize DNA by administration of folic acid (J. Paul, personal communication).

These results lead to two conclusions: (*a*) RNA molecules newly synthesized on a DNA template do not regulate the early increase in template activity of cells stimulated to proliferate, and (*b*) DNA-dependent RNA synthesis is not necessary for the synthesis of those proteins required for the increase in template activity, which suggest that the stimulating factor(s) for DNA synthesis acts on cells at a post-transcriptional level, triggering the synthesis of gene regulator proteins from previously formed messages. This is in agreement with the observations of Stein and Baserga (20), who have shown that the increase in nuclear acidic protein synthesis occurring in parotid glands of mice 1 hr after treatment with isoproterenol is insensitive to actinomycin D.

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