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Increased RNA Synthesis in Nuclear Monolayers of WI-38 Cells Stimulated to Proliferate

(endogenous [WI-38] RNA polymerase/exogenous RNA polymerase/chromatin template activity/gene sites/DNA synthesis)

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ABSTRACT **Nuclear monolayers of WI-38 cells prepared by the method of Tsai and Green were used to determine RNA synthesis in isolated nuclei *in situ*. In nuclear monolayers, incorporation of [^{3}H]UTP into RNA is dependent on the presence of the other three nucleotide triphosphates and is abolished by actinomycin D. The extent of RNA synthesis under these conditions was measured in density-inhibited WI-38 human diploid fibroblasts at various intervals after cell proliferation was stimulated by a change of medium.**

RNA synthesis increases 15 min after the nutritional change and reaches a peak at 18 hr, which is also the peak of DNA synthesis. Thereafter RNA synthesis declines. Essentially similar results are obtained whether the endogenous RNA polymerase or a bacterial polymerase is used. Replacement of the stimulating medium by conditioned medium stops the increase in RNA synthesis that occurs in cultures subject to continuous stimulation. Finally, RNA synthesis in nuclear monolayers, using the endogenous RNA polymerase, occurs by chain elongation only, while re-initiation occurs with the bacterial RNA polymerase.

In confluent monolayers of chick, rodent, or human fibroblasts in which DNA synthesis and mitosis have all but ceased (1, 2), proliferation can be induced by applying appropriate stimuli, such as neuraminidase (3), insulin (4), proteolytic enzymes (5, 6), hyaluronidase (7), or cortisol (8), or by replacing the old medium with fresh medium containing 10% serum (9–12).

One of the earliest events observed in stationary WI-38 human diploid fibroblasts stimulated to divide by a nutritional change (12) is an increase in chromatin template activity which is detectable within one hour after stimulation (13) and is noticeable whether a homologous or a bacterial (*Escherichia coli*) DNA-dependent RNA polymerase is used (14). Although an increase in chromatin template activity has also been reported in several other situations in which quiescent cells are stimulated to proliferate (15–21), these findings are subject to criticism, ranging from the use of an exogenous polymerase (22–28) to the preparation of chromatin (29). Recently, new techniques have become available for measuring RNA synthesis which avoid the problem of the precursors' pool by using monolayers stripped of most of their cytoplasm (30, 31). Intact nuclei attached to a glass or plastic surface are capable of synthesizing RNA *in situ* and with their own endogenous RNA polymerase (30, 31). We have used one of these new methodologies to study the effect of nutritional changes on RNA synthesis in density-inhibited WI-38 cells. In addition, we have investigated the question of RNA chain elongation or initiation, the use of an exogenous (*E. coli*) RNA polymerase, and, finally, the relationship between RNA synthesis and the extent of cell proliferation.

MATERIALS AND METHODS

Chemicals. [^{14}C]dT (specific activity 54.7 mCi/mmole), [^{3}H]dT (specific activity 6.7 Ci/mmole), [^{3}H]UTP (specific activity 26.9 Ci/mmole), [^{14}C]ATP (specific activity 49.3 mCi/mmole), and [γ^{32}P]GTP (specific activity 14.8 Ci/mmole) were purchased from New England Nuclear Corp., Boston, Mass. ATP, GTP, UTP, CTP, calf-thymus DNA, and *E. coli* tRNA were obtained from Sigma Chemicals, St. Louis, Mo. Actinomycin D was purchased from Schwarz-Mann, Orangeburg, N.Y. All other chemicals were supplied by Mallinckrodt Chemical Works, St. Louis, Mo., and the Fisher Scientific Co., Pittsburgh, Pa., analytical grades being used where available.

Cell Culture. WI-38 human diploid fibroblasts (32) were routinely grown in 35 × 10-mm Falcon plastic tissue culture dishes in Eagle's basal medium (Associated Biomedics Systems Inc., Buffalo, N.Y.) supplemented with 10% fetal-calf serum (Flow Laboratories), 100 IU/ml of penicillin, 50 μg/ml of streptomycin, and [^{14}C]dT (0.005 μCi/ml). The latter was used to uniformly label DNA and thus correct for variations in cell loss during the assays.

The cell cultures were free of contamination by pleuropneumonia-like organisms, as checked by autoradiography. The cells were used for experiments 7 days after plating (33). The confluent monolayers were stimulated to proliferate by replacing the old medium with medium containing 10% (w/v) fetal-calf serum. Two controls were used: cultures in which the old medium was not changed and cultures in which it was replaced by fresh medium containing 0.3% fetal-calf serum.

Isolation of E. coli RNA polymerase. RNA polymerase was isolated from *E. coli* K 12, as described by Burgess (34), by DEAE-cellulose chromatography and two subsequent glycerol density gradient centrifugations.

Preparation of Nuclear Monolayer. After the medium was removed, the cell monolayer was washed twice with 2.0 ml of phosphate buffered-saline and then 1.5 ml of 0.5% Triton X-100 in assay buffer containing 5 mM $MgCl_2$, 6 mM KCl, 50 mM Tris·HCl (pH 7.4), 75 mM $(NH_4)_2SO_4$, and 25% glycerol; 1 mM dithiothreitol was added. After 3 min the assay buffer containing Triton X-100 was removed and the nuclear monolayer was washed twice with 2.0 ml of assay buffer. This technique, slightly modified from the original of Tsai

and Green (31), gave clean nuclei, stripped of their cytoplasm, as checked by light microscopy.

RNA Synthesis Assay. (a) Endogenous (WI-38) RNA polymerase: A solution (0.5 ml) containing 20 μCi of [^{3}H]UTP, 5 mM ATP, 2 mM GTP, 2 mM CTP, and 85 pmol of UTP in assay buffer (see above) was pipetted on the nuclear monolayer. After the mixture was incubated at 37° for the desired length of time, the reaction was terminated by addition of 25 μl of 0.5 M EDTA. The whole mixture was carefully aspirated and the monolayer was washed with ice-cold phosphate-buffered saline. The nuclear monolayer was removed by addition of 0.5 ml of 0.5% Na dodecyl sulfate in phosphate-buffered saline and precipitated with 2.0 ml of 10% trichloroacetic acid solution containing 40 mM sodium pyrophosphate and *E. coli* tRNA (500 μg/ml) as a carrier. The precipitate was collected on 2.4-cm Whatman glass-fiber filters GF/C (W. and R. Balston Ltd., England) and washed thoroughly with 10% trichloroacetic acid. The filters were placed in special containers and combusted in an Intertechnique Oxymat, where the ^{3}H and ^{14}C were collected in separate vials containing special scintillator solutions (see Oxymat Manual). Samples were than counted in a Packard Scintillation spectrometer.

(b) Exogenous (E. coli) RNA polymerase: The technique was the same as in *a* except that the incubation mixture contained in 0.5 ml: 20 μCi of [^{3}H]UTP, 10 μl of *E. coli* RNA polymerase, 20 μmol of Tris·HCl (pH 8), 2 μmol of $MgCl_2$, 0.5 μmol of $MnCl_2$, 6 μmol of 2-mercaptoethanol, and 0.2 μmol each of ATP, CTP, GTP, and UTP.

(c) Incorporation of [γ-^{32}P]GTP: The incorporation of GTP labeled in the γ-position was measured under assay conditions that favored either the action of endogenous or exogenous RNA polymerase (see *a* and *b* above). Unlabeled GTP was omitted from the nucleotide triphosphates mixture and replaced by 25 μCi (0.08 μmole/mCi) of [γ-^{32}P]GTP.

In all these experiments, the incorporation of a radioactive precursor is expressed in pmoles per culture, after correction for any loss of cells during the assay.

RESULTS

RNA Synthesis in Nuclear Monolayers. RNA synthesis in nuclear monolayers of unstimulated WI-38 fibroblasts is linear in the first 15 min of incubation and levels off after 20 min (Fig. 1). There is negligible incorporation of [^{3}H]UTP into RNA when the unlabeled nucleotide triphosphates are omitted from the incubation mixture (Fig. 1). Actinomycin D (10 μg/ml) also inhibited by more than 90% the incorporation of [^{3}H]UTP into acid-precipitable material (not shown), thus confirming the claim of Tsai and Green (31) that nuclear monolayers synthesize *bona fide* RNA and not homopolymers.

RNA synthesis was also investigated in nuclear monolayers, with an exogenous RNA polymerase (*E. coli*). Under the conditions favoring the bacterial RNA polymerase there is negligible incorporation of [^{3}H]UTP into RNA by the endogenous RNA polymerase (Table 1).

RNA Synthesis after Nutritional Changes. RNA synthesis in nuclear monolayers of confluent monolayers of WI-38 cells at various intervals after nutritional changes is shown in Fig. 2. RNA synthesis begins to increase 15 min after medium change, has its steepest increase within the first hour, and reaches a maximum at 18 hr, which is also the peak of DNA

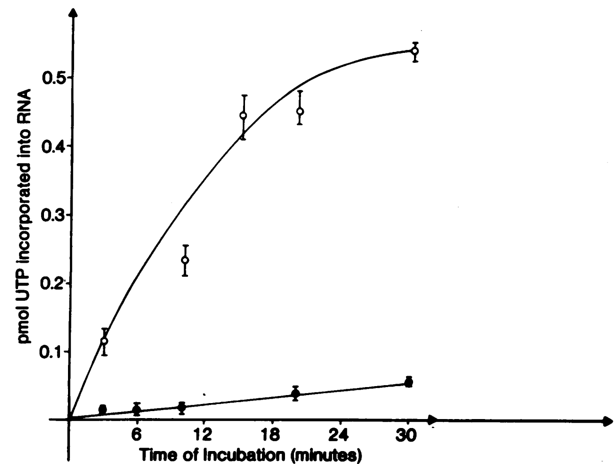

FIG. 1. Incorporation of [^{3}H]UTP into RNA by nuclear monolayers of stationary, unstimulated WI-38 human diploid fibroblasts. Cells were grown and RNA synthesis was assayed as described in *Methods*. (O——O) Incorporation of [^{3}H]UTP into RNA in the presence of all nucleotide triphosphates. (●——●) Incorporation of [^{3}H]UTP into RNA in the absence of the non-radioactive nucleotide triphosphates. Ordinate values are pmole per culture, after correction for cell loss.

synthesis (13). Thereafter, RNA synthesis declines slightly. It should be noted that 10 min after stimulation RNA synthesis is not increased (this point had to be omitted from Fig. 2). Even 0.3% fetal-calf serum in fresh medium caused an increase in RNA synthesis (Fig. 2) which, at 1 hr, was almost indistinguishable from the stimulation caused by 10% fetal-calf serum in fresh medium. At 6 hr, however, [^{3}H]UTP incorporation into RNA of cells in 0.3% fetal-calf serum levels off and remains stationary throughout the experimental period (25 hr). While 10% fetal-calf serum causes a 20-fold stimulation of DNA synthesis in confluent WI-38 (as measured by [^{3}H]dT incorporation 21 hr after stimulation), 0.3% fetal-calf serum causes a 4-fold stimulation (not shown). If serum is omitted from the fresh medium or if amino acids are added to the old medium, RNA synthesis is not increased above the level of cultures left in the medium originally used for plating (not shown). Neither of these two changes causes stimulation of cell proliferation in WI-38 cells (33).

TABLE 1. *Incorporation of [^{3}H]UTP into RNA by nuclear monolayers using exogenous (E. coli K 12) RNA polymerase*

	pmol of [^{3}H]UTP incorporated into RNA	
Min of incubation	Exogenous (*E. coli*) polymerase	Endogenous polymerase
5 (U)	0.030 ± 0.008	0.002 ± 0.001
15 (U)	1.42 ± 0.41	0.009 ± 0.001
25 (U)	1.45 ± 0.35	0.018 ± 0.003
15 (0.3%)	1.90 ± 0.28	—
15 (10%)	2.42 ± 0.37	—

RNA was assayed as described in *Methods*. Background incorporation was subtracted from the values given. *U* = unstimulated confluent monolayers; *0.3%* = 3 hr after stimulation with fresh medium plus 0.3% (w/v) serum; *10%* = the same with 10% serum.

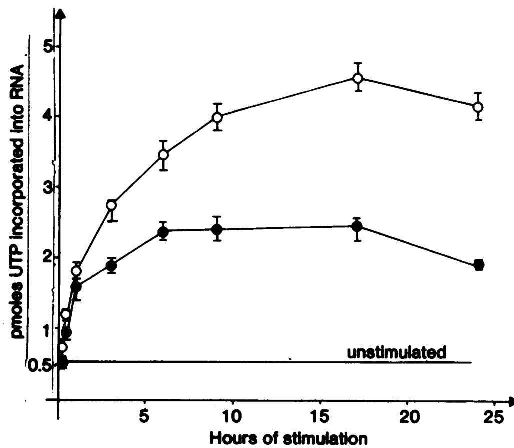

FIG. 2. RNA synthesis in nuclear monolayers of WI-38 cells at various intervals after stimulation to proliferate. Unstimulated cells were used as controls (——). Cells stimulated with 10% fetal-calf serum in fresh medium (O——O). Cells stimulated with 0.3% fetal-calf serum in fresh medium (●——●). Every point is the average of at least three independent experiments; the values given within the first hour are averages of 10 independent experiments. Incorporation of [^{3}H]UTP was determined as described in *Methods;* incubation time with the nucleotide triphosphate mixture was 15 min.

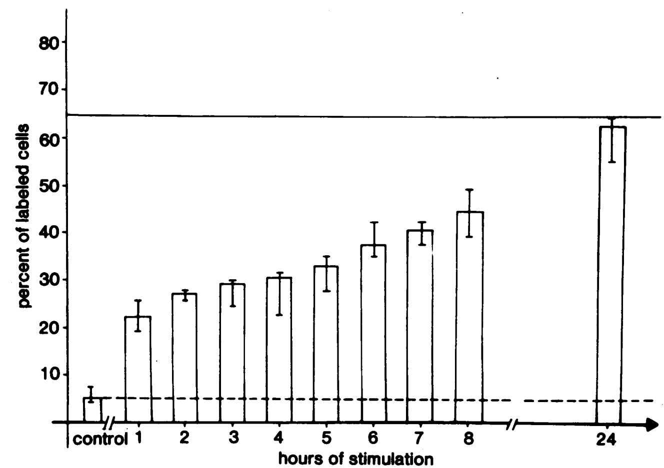

FIG. 3. Effect of duration of stimulation on the extent of cell proliferation. WI-38 diploid human fibroblasts were grown on glass cover slips in Falcon plastic tissue culture dishes (60 × 15 mm). On the seventh day after plating, the medium was removed and the cultures were washed twice with phosphate-buffered saline. Then fresh medium containing 10% fetal-calf serum was added and kept for the time indicated on the *abscissa.* Thereafter, this medium was replaced by conditioned medium, i.e., with old medium previously removed. After 8 hr, 1 μCi/ml of [^{3}H]dT was added to all cultures. All cultures were terminated after another 16 hr, and the cells were prepared for autoradiography as described by Baserga and Malamud (35).

The increase in RNA synthesis after stimulation with 10% fetal-calf serum in fresh medium is detectable in nuclear monolayers even when an exogenous *E. coli* RNA polymerase is used (Table 1).

Correlation Between RNA Synthesis and Stimulation of Cell Proliferation. To determine whether or not the increase in RNA synthesis may be correlated with the number of cells stimulated to proliferate, confluent monolayers of WI-38 fibroblasts were stimulated with fresh medium containing 10% fetal-calf serum for periods of time varying from 1 to 24 hr. After the desired period of stimulation, the stimulating medium was replaced by conditioned medium, and the number of cells entering DNA synthesis was determined by autoradiography (Fig. 3). In stationary WI-38 monolayers, approximately 5% of the cells are synthesizing DNA. When stimulated for 1 hr, 22.5% of the cells enter DNA synthesis after the usual lag period, the increase corresponding to the increase in RNA synthesis occurring within 1 hr after stimulation (Fig. 2). Thereafter, the percentage of stimulated cells rises steadily with increasing length of stimulation, reaching a maximum of 65% with continuous stimulation (Fig. 3). This again parallels the increase in RNA synthesis in nuclear monolayers. If there is indeed a correlation between increased RNA synthesis and stimulation of cell proliferation, one could predict that a 1-hr stimulation (causing only 22% of cells to enter DNA synthesis) should result in an early increase in RNA synthesis, rapidly leveling off. This prediction is borne out by the experiments described in Fig. 4. WI-38 cells were treated for 1 hr with 0.3% or 10% fetal-calf serum in fresh medium. The stimulating medium was then replaced by conditioned medium. Control cells were allowed to remain in the growth-promoting medium.

An increase in RNA synthesis in the first hour was found with both 10% and 0.3% fetal-calf serum. However, the cultures that were transferred back to conditioned medium did not show any further increase in RNA synthesis at later times.

Determination of Initiation Sites with [γ-^{32}P]GTP. The number of RNA chains initiated by RNA polymerase can be determined with [γ^{32}P]GTP and [γ-^{32}P]ATP (36). It has been shown, however, that [γ-^{32}P]ATP incorporation is not accurate when chromatin or nuclei are used, because there are additional interactions between ATP and chromosomal proteins, not related to the action of RNA polymerase itself (37). We, therefore, studied initiation in nuclear monolayers using only [γ-^{32}P]GTP. The results in Fig. 5 show that there is no appreciable initiation when endogenous RNA polymerase is used. Under assay conditions favoring the exogenous RNA polymerase, however, there is measurable incorporation of [γ-^{32}P]GTP into RNA. A 3-hr stimulation with 10% fetal-calf serum causes an increase in [γ-^{32}P]GTP incorporation using the *E. coli* RNA polymerase. After a 15-min incubation, there is a 55% increase in [γ-^{32}P]GTP incorporation by nuclear monolayers of cells stimulated for 3 hr, compared to unstimulated cell cultures. The actual values, in pmole of [γ-^{32}P]GTP incorporated per culture, were 0.451 ± 0.013 for stimulated and 0.289 ± 0.011 for unstimulated cultures.

DISCUSSION

It has been previously reported from this laboratory that in confluent monolayers of WI-38 human diploid fibroblasts stimulated to proliferate by fresh medium containing 10% fetal-calf serum there is a prompt increase in the template activity of chromatin isolated from the stimulated cells (13, 33). The results presented in this paper confirm by a different methodology that there is an increased gene activity in quiescent WI-38 cells stimulated to proliferate. The methodology used in this paper is a slight modification of the one proposed by Tsai and Green (31) and seems to measure the extent of RNA synthesis in monolayers of nuclei *in situ.* The advantage of this technique is that it avoids the problem of

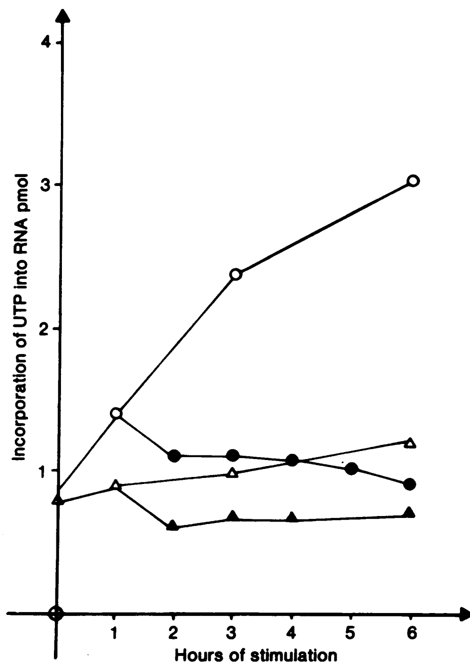

FIG. 4. RNA synthesis in nuclear monolayers after a 1-hr stimulation. WI-38 cells were grown as described. On the seventh day after plating, the old medium was removed and the cells were stimulated with 10% fetal-calf serum in fresh medium (○——○), or with 0.3% fetal-calf serum in fresh medium (△——△). After 1 hr of stimulation, the stimulating medium was removed from half of the cell cultures, the cells were washed twice with phosphate-buffered saline, and conditioned medium was again added. The remaining half of the cell cultures were left in the stimulating medium. (○——○) Cells stimulated by 10% fetal-calf serum in fresh medium; (●——●) cells stimulated for 1 hr by 10% fetal-calf serum in fresh medium followed by conditioned medium; (△——△) cells stimulated with 0.3% fetal-calf serum in fresh medium; (▲——▲) cells stimulated with 0.3% fetal-calf serum in fresh medium and followed by conditioned medium.

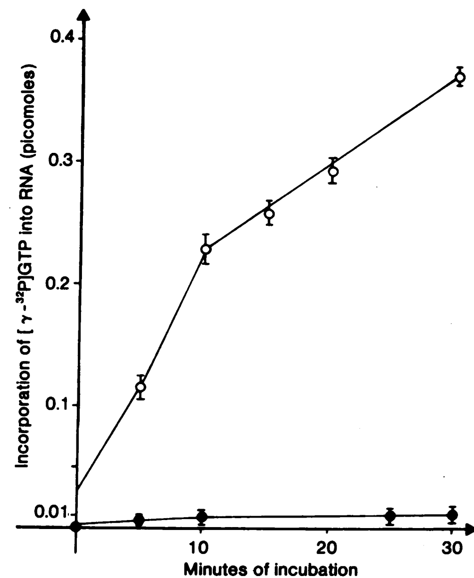

FIG. 5. Incorporation of [γ-^{32}P]GTP into RNA chain termini in nuclear monolayers of WI-38 cells. Incorporation was assayed in stationary unstimulated cell cultures as described in *Methods*. (●——●) Incorporation of [γ-^{32}P]GTP under conditions favoring the endogenous (WI-38) RNA polymerase (method *a*). (○——○) Incorporation of [γ-^{32}P]GTP in the presence of exogenous (*E. coli* K 12) RNA polymerase (method *b*).

the precursors' pool and of the changed uptake of the radioactive precursor, which can greatly alter measurements of RNA synthesis in whole cells (9, 38). In agreement with Tsai and Green (31), we find that the incorporation of a radioactive nucleotide triphosphate into nuclear monolayers depends on the presence of the other three nucleotide triphosphates and is inhibited by actinomycin D. Since, as mentioned above, an increase in chromatin template activity has been reported in several other situations in which quiescent cells are stimulated to proliferate (15–21), whether a bacterial or an homologous RNA polymerase is used (14, 21), the present results strongly support the validity of the previous findings, as well as of other reports on increased RNA synthesis in growing cells in culture obtained by determining the incorporation of radioactive precursors into whole cells (39, 40).

There are, however, differences between the results obtained with the standard chromatin template activity assay (13, 33) and the present methodology. The increase in chromatin template activity in WI-38 cells was detectable within 1 hr after stimulation, but it promptly reached a plateau and remained at the same level until the eighth hour, after which there was a further increase between 8 and 12 hr. With the technique of nuclear monolayers, the increase in RNA synthesis is detectable within 15 min after stimulation and it increases steadily up to the 18th hour after the nutritional change. At variance with the results of Mauck and Green (41) with 3T6 cells, we do find a lag between the application of the stimulus and the first detectable increase in RNA synthesis. The discrepancy between the results of Mauck and Green (41) and our own is probably due to the different type of cells, and as previously demonstrated, also to the standard chromatin template activity assay (33). The difference between the increase in chromatin template activity and increased RNA synthesis in nuclear monolayers of WI-38 cells could, instead, be due to the fact that the two methodologies may measure slightly different phenomena. While Todaro *et al.* (9) had already observed that it was necessary for confluent monolayers of 3T3 cells to remain in contact with fresh serum for at least 3 hr to obtain any appreciable stimulation, it was Bürk (42) who demonstrated in BHK cells that the number of cells stimulated to enter DNA synthesis was proportional to the length of stimulation. Our data confirm Bürk's results (42) that the number of cells stimulated to effect the transition from G_0 to S is proportional to the length of the application of the nutritional stimulus. In addition, the increase in RNA synthesis in nuclear monolayers is related to the number of stimulated cells. This is confirmed by the fact that the increased RNA synthesis in nuclear monolayers plateaus after 1 hr when quiescent cells are stimulated for a period of only 1 hr, and also by the results obtained with 0.3% fetal-calf serum.

Our results do not offer any evidence concerning the type of RNA whose synthesis is increased when quiescent cells are stimulated to proliferate. Mauck and Green (41), using 3T6 cells, have stated that only ribosomal RNA synthesis is increased after stimulation. However, subsequent studies from the same laboratory, using whole cells, have shown that both

rRNA and mRNA are increased in growing cells (43). Our data do not even indicate whether there is an increased synthesis of RNA or a decreased breakdown of the RNA synthesized by quiescent cells. However, using the technique of Cedar and Felsenfeld (37), Hill and Baserga (44) have demonstrated an increased number of binding sites of *E. coli* RNA polymerase in chromatin isolated from WI-38 cells stimulated to proliferate. Similar results have been obtained by Cox *et al.* (45) with chromatin of chick oviduct, and more recently Gross and Pogo (46) reported a decrease in the number of binding sites for *E. coli* RNA polymerase in starved yeast cells. Our data also show that in nuclear monolayers there is elongation of RNA chains, but no re-initiation (with the endogenous polymerase), confirming previous reports on isolated nuclei (47, 48). Together with the results presented here on the incorporation of [γ-^{32}P]GTP with the bacterial RNA polymerase, they seem to indicate that in WI-38 cells stimulated to proliferate there is an increased number of gene sites available for transcription. Finally, the facts that an exogenous bacterial *E. coli* RNA polymerase can also be used to measure RNA synthesis in nuclear monolayers, and that the increased RNA activity in WI-38 cells stimulated to proliferate can be demonstrated with the bacterial polymerase, open up the possibility of manipulating the nuclear milieu of quiescent cells in order to identify the macromolecules responsible for the increased gene activity in cells stimulated to proliferate.

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