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Template Activity of Chromatin during Stimulation of Cellular Proliferation in Human Diploid Fibroblasts

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1. Contact-inhibited confluent monolayers of WI-38 human diploid fibroblasts can be stimulated to divide by replacing the medium with fresh medium containing 30% foetal calf serum. 2. Of the cells 40–75% are stimulated to divide with a peak DNA synthesis between 15 and 21h and a peak mitotic index between 28 and 30h after stimulation. 3. In the first 12h before the initiation of DNA synthesis there is a biphasic increase in the incorporation of [^{3}H]uridine into RNA of whole cells. 4. This is paralleled by a similar biphasic stimulation of chromatin template activity measured *in vitro* in a system in which purified cell chromatin is incubated with an exogenous RNA polymerase isolated from *Escherichia coli*. 5. The changes in chromatin template activity are believed to represent activation of the genome, with more sites available for RNA synthesis, and to account almost entirely for the changes in RNA synthesis occurring in the whole cell.

Confluent monolayers of the WI-38 strain of human diploid fibroblasts can be stimulated to initiate DNA synthesis and subsequently to divide by a change of medium or addition of fresh serum (Rhode & Ellem, 1968; Wiebel & Baserga, 1969).

It has been shown in this laboratory (Wiebel & Baserga, 1969) that stimulation of non-dividing monolayers of WI-38 fibroblasts initiates changes in the rates of RNA and protein synthesis, which precede the onset of DNA synthesis occurring at about 12h after stimulation. Further, it has been shown (Rovera & Baserga, 1971) that these changes in protein and RNA synthesis are associated with or preceded by alterations in the rate of synthesis and turnover of non-histone chromosomal proteins, including those acidic nuclear proteins that are tenaciously bound to DNA (residual fraction). These changes in the metabolism of cellular RNA, total proteins and chromosomal proteins have been interpreted in this as well as in other models of stimulated DNA synthesis to indicate that the stimulation of DNA synthesis is mediated through the derepression of genes that regulate the synthesis of macromolecules necessary for the orderly duplication of the genome (Baserga, 1968; Allfrey, 1970).

In this paper we report that activation of the genome, as measured by an increase in the template activity of chromatin incubated with an exogenous RNA polymerase, appears to be an early phenomenon after stimulation of the cells. Further, the template activity of isolated chromatin *in vitro* during the pre-replicative phase appears to parallel the changes occurring in the incorporation of [^{3}H]uridine into the RNA of whole cells. It is postulated that the increased template activity is the major mechanism that the cells use to increase the rate of RNA synthesis.

MATERIALS AND METHODS

Chemicals. [*Me*-^{3}H]Thymidine (sp. radioactivity 14.1 Ci/mmol) was purchased from Amersham Searle, Arlington Heights, Ill., U.S.A. [5-^{3}H]Uridine (sp. radioactivity 25.9 Ci/mmol), [8-^{14}C]ATP (sp. radioactivity 54 mCi/mmol) and [^{3}H]leucine (sp. radioactivity 38 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. ATP, CTP, GTP and UTP were from Calbiochem, Los Angeles, Calif., U.S.A. All other chemicals were of reagent grade.

Cell culture. WI-38 human diploid fibroblasts (Hayflick & Moorhead, 1961) from Flow Laboratories, Rockville, Md., U.S.A., were plated in 1-litre Blake bottles as described by Rovera & Baserga (1971). At 7 days after plating each bottle contained approx. 7×10^6 cells. At that time the monolayers were confluent. The cells were stimulated to proliferate by replacing the old medium with fresh medium (Rovera & Baserga, 1971) containing 30% foetal calf serum (Flow Laboratories).

Radioautography. Cells were grown on cover-slips in small Petri dishes as described by Wiebel & Baserga (1969). They were stimulated with fresh medium containing 30% serum and 1 μCi of [*Me*-^{3}H]thymidine/ml was added to the medium. Individual cultures were arrested at the indicated times (Fig. 2) by washing the cells with ice-cold medium containing an excess of unlabelled thymidine. The cells were fixed in Carnoy's fixative (Baserga & Malamud, 1969) and radioautographs

were prepared as described by Baserga (1967). The emulsion used was Eastman Kodak NTB and the exposure time was 5 days. The percentage of labelled cells and the mitoses after metaphase block with 0.2 μg of Colcemid (*N*-deacetyl-*N*-methylcolchicine) (CIBA)/ml were determined on a total of 6000 cells in three different slides for each point.

Uptake of [^{3}H]*uridine into whole cells and its incorporation into RNA.* At various times after stimulation the medium was replaced with prewarmed Basal Eagle's Medium in Hanks balanced salt solution without serum and containing 1 μCi of [^{3}H]uridine/ml. The cells were incubated at 37°C for 30 min and then washed with ice-cold phosphate-buffered saline (Merchant, Kahn & Murphy, 1964). RNA was extracted by the method of Scott, Fraccastoro & Taft (1956) and determined by the two-wavelength modification of Fleck & Munro (1962) and Tsanev & Markov (1960). The radioactivity incorporated into RNA was determined by counting the radioactivity of 1 ml of the extract in a Triton–toluene 'cocktail' (Patterson & Green, 1965) in a Packard liquid-scintillation spectrometer at 35% efficiency. The uptake of [^{3}H]-uridine by whole cells was determined by counting the radioactivity of a portion of the acid-soluble fraction.

Isolation of chromatin. Chromatin was isolated, with minor modifications, by the method of Marushige & Bonner (1966). All operations were carried out at 4°C. The medium was decanted and the cells, washed once with the phosphate-buffered saline (Merchant *et al.* 1964), were harvested from the bottles by scraping with a rubber 'policeman'. The cells from two bottles (1.4×10^7) were pooled and homogenized with 40 strokes of a tight-fitting Dounce homogenizer in 4 ml of 0.075 M-NaCl and 0.024 M-EDTA (disodium salt), pH 8.0. The homogenate was centrifuged at 1500*g* for 15 min in the Sorvall SS-34 rotor. The crude nuclear pellet was washed successively with 4 ml of the above saline–EDTA solution and 4 ml of 0.05 M-tris–HCl buffer, pH 8.0 (at 4°C), and each time was collected by centrifugation at 1500*g* for 15 min. The washed nuclear pellet was resuspended in 4 ml of 0.05 M-tris–HCl buffer, pH 8.0, and disrupted with 60 strokes of a tight-fitting Dounce homogenizer and sedimented at 18000*g* for 15 min in the Sorvall centrifuge. The homogenization and centrifugation were repeated once. The final sediment was resuspended by gentle homogenization in 1 ml of the 0.05 M-tris–HCl buffer, layered on 4 ml of 1.7 M-sucrose and centrifuged for 80 min at 37500 rev./min in a Spinco SW 50 rotor. Approx. 80% of the cellular DNA was recovered in the chromatin pellet. This pellet was resuspended in 2 ml of 0.01 M-tris–HCl buffer, pH 8.0 (at 4°C), and dialysed for 12 h against 500 vol. of the same buffer. DNA and proteins were determined by the methods of Burton (1956) and Lowry, Rosebrough, Farr & Randall (1951) respectively. The absorption spectrum of chromatin in the u.v. and the protein/DNA ratio in unstimulated chromatin were similar to those reported by Marushige & Bonner (1966) for liver chromatin (Fig. 1).

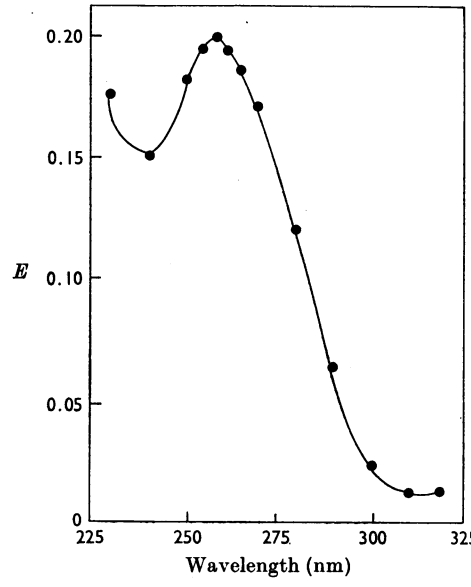

Fig. 1. Absorption spectrum in 0.01 M-tris–HCl buffer, pH 8.0, of chromatin isolated from unstimulated WI-38 fibroblasts.

Preparation of RNA polymerase. RNA polymerase was prepared from early exponential-phase *Escherichia coli* strain B purchased from General Biochemicals, Chagrin Falls, Ohio, U.S.A., by the method of Chamberlin & Berg (1962) as modified by Bonner *et al.* (1967). The enzyme was purified including the DEAE-cellulose column-chromatography step (fraction 4). Of this purified enzyme, 1 μg directed the incorporation of 2.8 nmol of [^{14}C]AMP/h into RNA. The ratio of RNA polymerase activity assayed with and without added DNA was greater than 400.

Assay of template activity. The complete incubation mixture for chromatin template activity for RNA synthesis contained in a final volume of 250 μl: 10 μmol of tris–HCl buffer, pH 8.0; 1 μmol of $MgCl_2$; 0.25 μmol of $MnCl_2$; 3 μmol of 2-mercaptoethanol; 0.1 μmol each of GTP, CTP and UTP; 0.1 μmol of [^{14}C]ATP (sp. radioactivity 1 μCi/μmol); chromatin containing up to 10 μg of DNA and 18 μg of fraction 4 *E. coli* RNA polymerase. Incubation was carried out at 37°C for 10 min. The reaction was stopped by addition of ice-cold 10% (w/v) trichloroacetic acid. The acid-insoluble material was collected by filtration through a Millipore filter and was washed four times with 5 ml of 10% trichloroacetic acid. The filters were dissolved in 1 ml of Cellosolve (2-ethoxyethanol) and their radioactivities were counted in 15 ml of Cellosolve–toluene (1:3, v/v) scintillation cocktail (Gilman, 1970) in a Packard liquid-scintillation spectrometer at 80% efficiency.

Ribonuclease activity of chromatin. Ribonuclease activity of the chromatin preparations was determined by incubating labelled ribosomal RNA with chromatin in the reaction mixture for RNA synthesis described above. Ribosomal RNA with a specific radioactivity of 890 d.p.m./μg was obtained from actively proliferating WI-38 cells by disrupting about 2×10^7 cells with a tight-fitting Dounce homogenizer after swelling the cells for 10 min in 2 ml of RSB medium (0.01 M-NaCl; 0.0015 M-$MgCl_2$; 0.01 M-tris–HCl buffer, pH 7.4). Immediately before homogenization 40 μl of diethyl pyrocarbonate was added (Abadom & Elson, 1970). The homogenate was sedimented at 4°C

at 27000*g* for 15min. Sodium deoxycholate was added to the supernatant to a final concentration of 1%, and the ribosomes were collected by centrifugation at 100000*g* for 90min in a Spinco 40 rotor. The ribosomes were extracted with an equal volume of phenol and 0.14M-NaCl–0.01M-EDTA (disodium salt), pH6.5, at 4°C as described by Rovera, Berman & Baserga (1970). Ribonuclease activity was determined by incubating ribosomal RNA for 10min with or without chromatin in the complete assay mixture described above, minus the [^{14}C]ATP, and expressed as recovery of acid-insoluble radioactivity.

Proteolytic activity of chromatin. Chromosomal proteins were prelabelled for 30min with 1μCi of [^{3}H]leucine/ml as described by Rovera & Baserga (1971), and the chromatin was extracted as described above. Of the dialysed chromatin suspension 0.6ml was incubated at 37°C in the incubation mixture for chromatin template activity described above, minus RNA polymerase and [^{14}C]ATP, in a final volume of 1.5ml. Samples (0.1ml) were removed at zero time and at 10min, 30min, 1h and 3h. The samples were precipitated with 10% trichloroacetic acid; collected on Millipore filters, washed and their radioactivities counted in the Cellosolve–toluene scintillation mixture described above. Proteolytic activity was expressed as the recovery of acid-insoluble radioactive protein.

RESULTS

Stimulation of DNA synthesis. At 7 days after plating, confluent monolayers of WI-38 cells show a very low rate of DNA synthesis and mitosis, with 0.1–0.5% of the cells being labelled by a 20–25h exposure to [^{3}H]thymidine. Replacement of the exhausted medium with fresh medium containing 30% foetal calf serum stimulated 40–75% of the cells to begin DNA synthesis within 12–20h (Fig. 2). The cells start dividing 24h after stimulation with fresh medium, and the cumulative mitotic index reaches a peak at 33h. The use of 30% serum has increased the percentage of cells stimulated over that previously reported from this laboratory with the use of 10% serum (Wiebel & Baserga, 1969). The percentage of cells that synthesize DNA and subsequently divide varies with the batch of foetal calf serum and with the age (number of generations) of the culture. Cultures older than 30 generations show poor stimulation and are not used as a routine. With certain batches of serum, up to 75% of the cells were stimulated.

Synthesis of RNA in whole cells in the pre-replicative period. In these experiments, the rate of RNA synthesis was estimated from the specific radioactivity of total RNA after a 30min pulse with [^{3}H]uridine (see the Materials and Methods section). Table 1 shows the specific radioactivity of total RNA during the first 12h after stimulation. The uptake of [^{3}H]uridine into the acid-soluble fraction is also shown. Within 1h after stimulation there is a 50% increase in the incorporation of [^{3}H]uridine into RNA. This increased incorporation remains

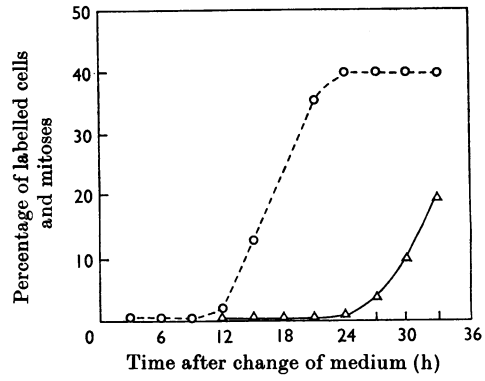

Fig. 2. Percentage of cells labelled by [^{3}H]thymidine and mitotic index after stimulation of WI-38 cells by change of medium. [^{3}H]Thymidine was added at zero time and the percentage of labelled cells determined by radioautography as detailed in the Materials and Methods section. The percentage of mitoses was determined after metaphase block with 0.2μg of Colcemid/ml added at 18h after stimulation. ○, Percentage of cells labelled; △, percentage of mitoses.

Table 1. *Uptake of* [^{3}H]*uridine into cells and its incorporation into RNA at various intervals after stimulation by changing the medium*

For details see the text. Results are means of duplicates ±S.D.

Time after change of medium (h)	Incorporation of [^{3}H]uridine into RNA (d.p.m./μg of RNA)	Radioactivity in acid-soluble fraction (d.p.m./μg of DNA)
0	702 (±78)	504 (±132)
1	1026 (±57)	417 (±60)
5	1143 (±135)	504 (±12)
8	1140 (±144)	471 (±111)
12	1734 (±249)	870 (±186)

constant through the 8th hour after stimulation. By 12h there is a further 70% increase in the incorporation of [^{3}H]uridine into RNA. As shown in Table 1 the uptake of [^{3}H]uridine into cells during the first 12h is not significantly different from the uptake by unstimulated cells. The incorporation of [^{3}H]uridine into RNA therefore appears to reflect the rate of RNA synthesis. We conclude that there is a biphasic increase in the rate of RNA synthesis. at 1 and 12h after stimulation of cells. The magnitude of these changes in any given experiment depends on the number of cells that subsequently divide and varies, as mentioned above, with the age of the cultures and the batch of foetal calf serum.

Template activity of chromatin from stimulated and

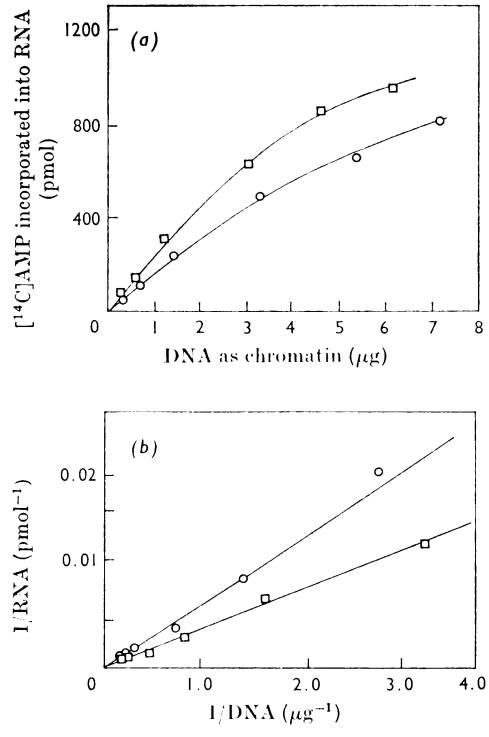

Fig. 3(*a*) Template activity of chromatin from unstimulated WI-38 cells and from cells 1 h after stimulation. Chromatin was isolated and its template activity determined as detailed in the Materials and Methods section. Each point was assayed with 18 μg of fraction 4 *E. coli* RNA polymerase. ○, Unstimulated; □, stimulated for 1 h. (*b*) Double-reciprocal plot of the results in (*a*).

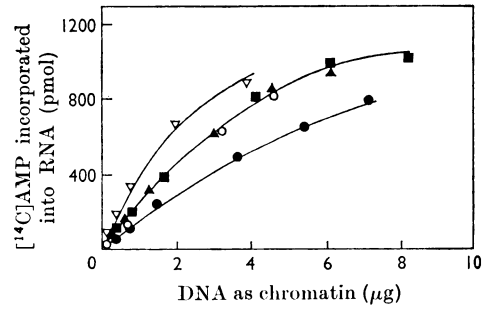

Fig. 4. Template activity of chromatin from unstimulated cells and from cells 1, 5, 8 and 12 h after stimulation. Each point was assayed with 18 μg of fraction 4 RNA polymerase as described in the Methods and Materials section. ●, Unstimulated; ■, 1 h; ▲, 5 h; ○, 8 h; △, 12 h.

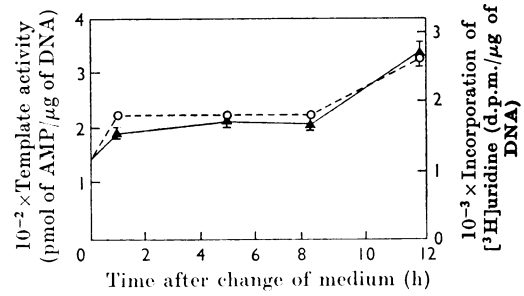

Fig. 5. Comparison of the incorporation of [^{3}H]uridine into RNA and template activity of chromatin of WI-38 cells at various intervals after stimulation by a change of medium. Incorporation of [^{3}H]uridine into RNA *in vivo*/μg of DNA (▲) was determined by a modification of the method of Scott *et al.* (1956) detailed in the Materials and Methods section. Chromatin template activity *in vitro*/μg of DNA (○) is derived from the results in Fig. 3 by using the values obtained with 1 μg of chromatin DNA.

unstimulated cells. The template activity of chromatin with an exogenous (neutral) *E. coli* RNA polymerase was investigated in unstimulated cells and in stimulated cells in the first 12 h after stimulation. Fig. 3(*a*) shows that within 1 h of stimulation there is an increase in the template activity of chromatin isolated from stimulated cells. Since it requires prohibitive amounts of the *E. coli* enzyme to run the assay under conditions of saturation with RNA polymerase, the results are presented as a chromatin-concentration curve with a fixed amount of RNA polymerase. The double-reciprocal plot of the same results in Fig. 3(*b*) confirms the stimulation of template activity of chromatin isolated from cells 1 h after stimulation. The two chromatin preparations, although showing different rates of RNA synthesis at sub-saturating chromatin concentrations, have the same $V_{max.}$ This we interpret to mean that the affinity of the enzyme-binding sites on the chromatin for RNA polymerase is the same and that there are simply more sites available for RNA synthesis in the chromatin of stimulated cells although changes in existing sites cannot definitely be ruled out. A specific alteration in the binding site for the polymerase should be reflected in a different $V_{max.}$.

Fig. 4 compares the template activity of chromatin isolated from unstimulated cells and from cells 1, 5, 8 and 12 h after stimulation. After the increase occurring at 1 h, template activity remains elevated until the 12th hour, when it increases further.

Fig. 5 compares incorporation of [^{3}H]uridine into RNA and chromatin template activity at various intervals after stimulation. The template activity of the isolated chromatin parallels almost exactly the changes in incorporation of [^{3}H]uridine into RNA. In both cases, there is a clearly apparent biphasic stimulation with increases occurring be

tween 0 and 1h and 8 and 12h. This confirms the fidelity of transcription *in vitro* and implies that the changes observed in the whole cell may be due entirely to changes in the chromatin.

Ribonuclease and proteolytic activity of chromatin. Two controls are necessary before the increased template activity of chromatin from stimulated cells can be directly related to the changes in the whole cell. The differences in chromatin template activity between stimulated and unstimulated cells could reflect differences in ribonuclease (Morgan & Bonner, 1970). Table 2 shows that there is no detectable ribonuclease activity in chromatin from unstimulated cells and from cells 1h after stimulation. Labelled ribosomal RNA from unstimulated WI-38 cells was added to complete chromatin-template assay mixtures minus [^{14}C]ATP. The recovery of acid-insoluble radioactivity in 10min was identical in the samples without chromatin and with unstimulated and stimulated chromatin. Differences in ribonuclease activity cannot therefore account for the differences in template activity in chromatin from stimulated and unstimulated cells.

It has been shown (Panyim, Jensen & Chalkley, 1968; Bartley & Chalkley, 1970) that isolated nucleoprotein complexes contain a histone protease activity. By hydrolysing and solubilizing histones, this protease could alter the template activity of chromatin. Table 3 shows that chromatin preparations from unstimulated and stimulated cells contain proteolytic activity. However, there are no differences in the amount of proteolytic activity among the various chromatin preparations. The loss of radioactivity from the acid-precipitable fraction in the first 10min of incubation was 11% in chromatin from unstimulated cells, 9% in chromatin from cells 1h after stimulation, and 11% in chromatin from cells stimulated for 12h. After 1h of incubation, 76% of the original radioactivity was recovered from chromatin of unstimulated cells, 71% from chromatin of cells stimulated for 1h, and 78% from chromatin of cells stimulated for 12h.

Table 2. *Ribonuclease activity of chromatin from unstimulated cells and from cells* 1h *after stimulation*

Ribonuclease activity is measured as acid-insoluble radioactivity recovered from a 10min incubation containing labelled ribosomal RNA, chromatin and the complete template-activity assay minus [^{14}C]ATP. Results are means of duplicates ±S.D.

	Radioactivity (d.p.m.)
Minus chromatin	15913±500
Unstimulated chromatin (72μg of DNA)	14483±1500
Chromatin 1h after stimulation (65μg of DNA)	14713±1500

DISCUSSION

Confluent monolayers of WI-38 fibroblasts can be stimulated to divide by replacing the medium with fresh medium containing 30% foetal calf serum. During the 12h pre-replicative period after stimulation of cells there is a biphasic activation of both RNA synthesis and chromatin template activity. Systems like the template assay used here *in vitro* have been shown in a number of laboratories (Bekhor, Kung & Bonner, 1969; Gilmour & Paul, 1969; Smith, Church, & McCarthy, 1969; Tan & Miyagi, 1970) to produce RNA indistinguishable from RNA produced *in vivo*. Although physical changes in chromatin under different experimental conditions have not been ruled out, this fidelity of transcription would imply that they are not significant. Quantitative chromatin template activity is believed to be directly proportional to the RNA synthesis rate *in vivo*. The absence of differences in ribonuclease and proteolytic activity reported above lends further support to the belief that chromatin template activity actually reflects RNA synthesis *in vivo* both qualitatively and quantitatively. Therefore, we interpret the fact that

Table 3. *Proteolytic activity of chromatin preparations*

Chromatin prelabelled with [^{3}H]leucine was incubated at 37°C as described in the Materials and Methods section. Triplicate samples were taken at the times indicated and precipitated on Millipore filters with 10% trichloroacetic acid. Proteolytic activity (d.p.m./μg of DNA) is measured as acid-insoluble radioactivity recovered from the incubation mixture. Results are means of triplicates ±S.D.

	Proteolytic activity (d.p.m./μg of DNA)		
Time of incubation (min)	Non-stimulated cells	Cells stimulated for 1h	Cells stimulated for 12h
0	1003 (±34)	1143 (±26)	1075 (±19)
10	896 (±26)	1048 (±34)	953 (±41)
30	836 (±34)	953 (±22)	870 (±15)
60	771 (±19)	820 (±19)	836 (±26)

chromatin template activity parallels the change in rate of RNA synthesis to imply that the major mechanism accounting for the increased rate of RNA synthesis is an increased number of sites available *in vivo* for RNA transcription.

Stimulation of our cells failed to produce significant differences in the uptake of [^{3}H]uridine. This is in contrast with the stimulation of [^{3}H]-uridine uptake reported with addition of serum to contact-inhibited 3T3 cells (Cunningham & Pardee, 1969). Species differences probably account for these variations in uptake of [^{3}H]uridine.

An increased chromatin template activity before the initiation of DNA synthesis has been reported in other systems in which resting cells are stimulated to divide: in resting lymphocytes stimulated to divide by phytohaemagglutinin (Hirschhorn, Troll, Brittinger & Weissmann, 1969), in rat liver cells stimulated to divide by partial hepatectomy (Bannai & Terayama, 1969), and in oestrogen-stimulated rat uterus cells (Teng & Hamilton, 1969). In the latter system many of the changes preceding DNA synthesis may be related to hypertrophy of the existing cells, restoring them to the size and activity of those in the normal animal rather than to preparation for cell division (Mueller, Herranen & Servell, 1958). In the first system whole nuclei were used as the source of chromatin, and changes in ribonuclease activity could not be ruled out. In regenerating liver the changes in template activity were small, compared with the 10–12-fold increase that has been reported (Chaudhuri, Loi & Lieberman, 1967) in the rate of labelling of cytoplasmic RNA after partial hepatectomy. An explanation for this lack of correlation between template activity and the rate of labelling of cytoplasmic RNA in regenerating liver is the fact that cytoplasmic labelling predominantly reflects ribosomal RNA synthesis, whereas template activity is believed to reflect the entire spectrum of RNA molecules being transcribed at the time the chromatin is isolated. The percentage of this spectrum that is ribosomal RNA molecules may be quite small and large changes in the template for ribosomal RNA may therefore be obscured by the bulk of non-ribosomal RNA being transcribed *in vitro*.

This leads us to ask what percentage of the RNA synthesized *in vitro* is represented by ribosomal RNA in both the unstimulated chromatin assay and the one at 1 and 12h after stimulation of cells. It has been suggested (Lee, Vaughan & Abrams, 1970) that a stepped-up synthesis of ribosomes is a key event in mammalian cells during the transition from a state of mitotic inactivity to one of active growth. The burst of ribosome formation seen in many systems before the initiation of DNA synthesis (Chaudhuri *et al.* 1967; Tata, 1968; Lee *et al.* 1970; Sasaki & Baserga, 1970) could be the trigger for DNA synthesis in these systems. Our system, in which there is a biphasic stimulation of template activity which appears to determine a similar pattern in the rate of RNA synthesis in the whole cell, should enable us to test this hypothesis critically.

At present we suggest that replacement of the old medium with fresh medium containing 30% serum may alter the translational control of preformed messages for specific acidic proteins, as demonstrated in the isoproterenol-stimulated salivary gland (Stein & Baserga, 1970). The newly synthesized acidic proteins enter the nucleus and activate portions of the genetic template so that RNA molecules are transcribed at a faster rate. Whether new RNA molecules are made is not known at present. These RNA molecules in turn would supply the templates for proteins that initiate DNA synthesis directly or through control of ribosomal RNA synthesis.

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