Changes in the Number of Binding Sites for Ribonucleic Acid Polymerase in Chromatin of WI-38 Fibroblasts Stimulated to Proliferate

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1. When WI-38 human diploid fibroblasts form confluent monolayers, DNA synthesis and cell division almost completely cease. A change of medium causes these density-inhibited cells to proliferate and within 1 h after the application of the stimulus there is an increase in template activity of the chromatin isolated from stimulated cells. 2. The number of binding sites for *Escherichia coli* RNA polymerase was determined on chromatin from WI-38 cells by two different methods, i.e. incorporation of [³H]UTP into RNA in the absence of reinitiation, and incorporation of [γ -³²P]GTP into chain termini. 3. Both methods indicate that the capacity of chromatin to bind *E. coli* RNA polymerase is increased in WI-38 cells stimulated to proliferate. 4. The increase in the number of binding sites for *E. coli* RNA polymerase parallels the increase in chromatin template activity and suggests that the latter reflects an increase in the number of initiation sites, rather than an increase in the rate of transcription.

Confluent monolayers of human, rodent or chick fibroblasts, in which DNA synthesis and cell division have all but ceased (Stoker, 1962; Pontén, 1971), can be stimulated to proliferate again when the old medium is replaced by fresh medium containing 10% (w/v) serum (Todaro *et al.*, 1965; Yoshikura & Hirokawa, 1967; Rhode & Ellem, 1968; Temin, 1971). This is also true of WI-38 human diploid fibroblasts, in which replacement of the old medium with fresh medium plus 10% (w/v) serum causes as many as 80% of the cells to resume synthesis of DNA and divide (Wiebel & Baserga, 1969). One of the earliest events occurring after the nutritional change is an increase in template activity of chromatin, which is detectable within 1 h of stimulation (Farber *et al.*, 1971). This increase in chromatin template activity is apparent whether an exogenous *Escherichia coli* or an homologous HeLa-cell DNA-dependent RNA polymerase is used in the assay mixture (Farber *et al.*, 1972).

The increase in template activity of chromatin from stimulated cells may be attributed to (1) an increase in the number of binding sites for RNA polymerase, or (2) an increase in the rate of elongation of the RNA chains. Cedar & Felsenfeld (1973), in comparing the template activities of calf thymus DNA and calf thymus chromatin, have described methods that can distinguish between these two alternatives. The purpose of the present investigations was to determine the number of binding sites for *E. coli* DNA-dependent RNA polymerase and the rate of propagation of growing RNA chains in chromatin isolated from confluent monolayers of either stimulated or

unstimulated WI-38 fibroblasts. The number of binding sites capable of supporting chain initiation was determined by counting the number of growing RNA chains in an assay that allowed initiation, but not re-initiation, by RNA polymerase and, in part, by measuring the incorporation of [γ -³²P]GTP into chain termini (Cedar & Felsenfeld, 1973). The results indicate that chromatin from WI-38 cells stimulated to proliferate has a higher number of binding sites for *E. coli* RNA polymerase than does chromatin from control cells.

Materials and Methods

Chemicals

[¹⁴C]ATP (sp. radioactivity 48 mCi/mmol), [³H]-UTP (sp. radioactivity 30.2 Ci/mmol) and [γ -³²P]GTP (sp. radioactivity 11.6 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Non-radioactive ATP, CTP, GTP, UTP, bovine serum albumin and calf thymus DNA were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Highly purified *E. coli* K12 RNA polymerase (sp. activity 382 units/mg of protein) was purchased from Miles Laboratories, Elkhart, Ind., U.S.A. All other chemicals were supplied by Fisher Scientific Co., Pittsburgh, Pa., U.S.A., or Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A., AnalaR grades being used where available.

Cell culture

WI-38 human diploid fibroblasts (Hayflick & Moorhead, 1961) from Flow Laboratories, Rockville,

Md., U.S.A., were grown in plastic Falcon flasks (surface area 75cm²) in Basal Eagle's Medium (Associated Biomedics Systems Inc., Buffalo, N.Y., U.S.A.), supplemented with 10% (w/v) foetal calf serum (Flow Laboratories) and antibiotics as described by Rovera & Baserga (1971). At 7 days after plating, confluent monolayers were stimulated to proliferate by replacing the old medium with fresh medium containing 10% (w/v) foetal calf serum (Rovera & Baserga, 1971). Control cells were re-fed with fresh medium containing 0.3% (w/v) foetal calf serum, a procedure that causes only slight stimulation of cell proliferation in confluent monolayers of WI-38 fibroblasts (Rovera & Baserga, 1973).

Isolation of chromatin

Chromatin was prepared from a purified nuclear fraction by the method of Paul & Gilmour (1968) with slight modifications (Novi & Baserga, 1972). Chromatin was used unsheared except in one experiment (Table 1), when it was sheared in a VirTis '45' homogenizer, in the 2ml-capacity stainless-steel flanged cup, with a power input of 80V for 30s shearing time, as described by Duerksen & McCarthy (1971).

Assay of chromatin template activity

Chromatin template activity was determined as described previously (Farber *et al.*, 1971), by using *E. coli* RNA polymerase prepared by the method of Chamberlin & Berg (1962) as modified by Bonner *et al.* (1967). The enzyme was purified up to and including the DEAE-cellulose column-chromatography step (fraction 4) and had a 250-fold dependence on exogenous DNA.

Determination of binding sites for E. coli RNA polymerase

All assays were performed in duplicate with the inclusion of appropriate enzyme and substrate blanks, the values of which have been subtracted from the quoted results. In these experiments the highly purified *E. coli* K12 RNA polymerase purchased from Miles Laboratories was used.

(i) *Incorporation of [³H]UTP without re-initiation.* The procedure of Hyman & Davidson (1970) was followed as described by Cedar & Felsenfeld (1973), with the following minor modifications. RNA polymerase and chromatin were incubated at 37°C in a final volume of 0.5ml containing 10mM-Tris-HCl, pH7.9, 1mM-MnCl₂, 0.08mM-ATP, 0.08mM-GTP and 0.02mM-[³H]UTP (750c.p.m./pmol). Initiation was allowed to proceed for 15min before the addition of 0.16ml of 1.6M-(NH₄)₂SO₄ which prevents any

further chain initiation or re-initiation by enzyme molecules (Hyman & Davidson, 1970). Propagation in high salt was then started by the addition of 20μl of CTP (2.2mM) and 20μl of MgCl₂ (1.75mM). Incorporation into RNA was determined at various times after the commencement of propagation as described by Cedar & Felsenfeld (1973), except that the trichloroacetic acid-precipitated samples were collected on Millipore filters and washed with 4×5ml of 5% (w/v) trichloroacetic acid containing 0.01M-sodium pyrophosphate. The filters were dissolved in 1ml of ethylCellosolve (2-ethoxyethanol) and radioactivity was counted in 15ml of a Cellosolve-toluene scintillant (Gilman, 1970) in a Packard liquid-scintillation spectrometer at 30% efficiency. Koslov & Georgiev (1970), in determining the total amount of nucleotide triphosphates incorporated under these conditions, assumed it to be 3.45 times the incorporation of UTP, since calf thymus DNA contains 58% A+T: this factor has been used in the calculations listed in Table 1.

(ii) *Sucrose-gradient analysis.* For sucrose-gradient analysis duplicate 0.2ml portions of the final assay mixture [see (i) above] were adjusted to 0.5% (w/v) sodium dodecyl sulphate, 7.5mM-EDTA, pH7.0, and kept at 22°C for 1h before centrifugation as described by Cedar & Felsenfeld (1973). Ribosomal RNA, prepared from rat liver by the method of Wilson & Quincy (1969), was used as a molecular-weight marker to standardize the gradients. The average chain length (*n*) in nucleotides of the RNA was calculated in each fraction by using the gradient relationship $\log_{10} n = 2.10 \log_{10} S_{20,w} + 0.644$ (Hyman & Davidson, 1970).

(iii) *Incorporation of [^γ-³²P]GTP.* This was measured under assay conditions that allowed initiation but prevented re-initiation [see (i) above], by using unlabelled UTP (0.02mM) and 0.08mM-[^γ-³²P]GTP (1650c.p.m./pmol). The extent of incorporation after 35min propagation in high salt [0.4M-(NH₄)₂SO₄] was measured after precipitation of the entire assay mixture with HClO₄ as described by Maitra & Hurwitz (1965) with the following modifications. The acid-insoluble material, after thorough washing, was finally solubilized in 0.2M-NaOH and samples were counted for radioactivity, after acidification, in a Triton-toluene scintillant (Patterson & Greene, 1965).

(iv) *Titration of RNA polymerase with chromatin.* The method of Cedar & Felsenfeld (1973) was followed: 0.8 unit of *E. coli* K-12 RNA polymerase was titrated with various amounts of chromatin. After a 15min period of initiation, chain elongation was started with high salt, CTP and MgCl₂ and allowed to continue for 35min before the reaction was terminated. The final extent of [³H]UTP incorporation was used to evaluate the number of initiated RNA chains in each reaction mixture.

Results

Incorporation of UTP into RNA in high salt, after initiation in low salt

Cedar & Felsenfeld (1973), in their studies of the interaction of *E. coli* RNA polymerase with DNA and chromatin, utilized purified calf thymus DNA and sheared calf thymus chromatin. Chain initiation was allowed to take place at low salt concentration in an incubation mixture from which one of the nucleotide triphosphates, CTP, was omitted. After all sites capable of binding *E. coli* RNA polymerase were saturated, propagation of RNA chains was allowed to continue by adding $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 0.4M, MgCl_2 and CTP. The high salt concentration does not allow re-initiation by RNA polymerase, so that the number of growing RNA chains is an indication of the number of binding sites (Hyman & Davidson, 1970; Cedar & Felsenfeld, 1973). One of the criteria used by Cedar & Felsenfeld (1973) for achieving quantitative results in their experiments was that chromatin must be soluble in the incubation mixture. Our initial experiments were designed to determine whether their methods were also applicable to unsheared chromatin.

Preliminary results (not shown here) demonstrated that all sites on unsheared chromatin capable of binding *E. coli* RNA polymerase were saturated after an initiation period in low salt (without CTP) of 10min and that propagation in high salt was terminated 35min after the addition of CTP, MgCl_2 and $(\text{NH}_4)_2\text{SO}_4$. In our experiments to determine the incorporation of UTP into RNA we therefore chose an initiation time of 15min and a propagation time of 35min. The extent of UTP incorporation into RNA in high salt (after initiation in low salt) is shown in Table 1. Calf thymus DNA, sheared and unsheared chromatin from control WI-38 cells and unsheared chromatin from WI-38 cells 1h after stimulation by fresh serum were studied. Table 1 shows that under the conditions of these experiments the amount of

RNA polymerase is in excess, since doubling the enzyme concentration has essentially no effect on the incorporation of UTP. The data also demonstrate that the extent of UTP incorporation into RNA with sheared control chromatin as template is approximately one-tenth of that obtained with a calf thymus DNA template, confirming the findings of Cedar & Felsenfeld (1973). With unsheared control chromatin the incorporation of UTP into RNA is further decreased, to approx. 3% of the value with DNA. These data were reproducible under the experimental conditions used here, which differed only from those of Cedar & Felsenfeld (1973) in that the propagation time was slower, requiring 35min to reach a plateau.

Table 1 also demonstrated that there is a 40% increase in the incorporation of UTP into RNA when chromatin isolated from stimulated WI-38 cells replaces chromatin isolated from control cells. This difference is also shown in Fig. 1(b), which shows the kinetics of incorporation of UTP into RNA in 0.4M- $(\text{NH}_4)_2\text{SO}_4$ after initiation in low salt concentrations by chromatin isolated from either control cells or cells stimulated to proliferate. For control chromatin the rate of UTP incorporation during the propagation period is 0.6pmol/min for 2 μg of DNA as chromatin. The rate of UTP incorporation with the same quantity of chromatin from stimulated cells is 0.8pmol/min, an increase of 33%. The incorporation of UTP by this method is then compared with a standard assay for chromatin template activity (Farber *et al.*, 1971), for both control and stimulated cells (Fig. 1a).

The increase in chromatin template activity 1h after confluent monolayers of WI-38 cells are stimulated to proliferate confirms the earlier observations made in this laboratory (Farber *et al.*, 1971; Rovera *et al.*, 1971; Rovera & Baserga, 1973). In this particular case (Fig. 1a) the extent of increase in chromatin template activity in stimulated cells was 36% and thus paralleled the increase in the extent of UTP incorporation by stimulated chromatin in high salt, after initiation in low salt.

Table 1. Nucleotide incorporation into RNA in high salt after initiation in low salt, with DNA and chromatin as templates

Assay conditions are described in the Materials and Methods section. Total nucleotides incorporated were calculated as described in section (i), 'Incorporation of [^3H]UTP without re-initiation'. One unit of RNA polymerase was defined as that amount of enzyme catalysing the incorporation of 1 nmol of [^{14}C]ATP in 10min at 37°C into acid-insoluble material, in the presence of calf thymus DNA. DNA (2 μg) was used as template in these experiments, either as DNA or in chromatin.

Template	RNA polymerase (units)	UTP incorporation (pmol)	Total nucleotides incorporated (pmol)
DNA	4	274	944
	8	279	960
Sheared control chromatin	4	25.8	89
	8	27	93
Unsheared control chromatin	4	8.4	29
	8	9.0	31
Unsheared stimulated chromatin	4	11.6	40
	8	12.5	43

Fig. 1. (a) Template activity of chromatin from unstimulated control WI-38 cells and from WI-38 cells 1 h after stimulation and (b) incorporation of $[^3\text{H}]\text{UTP}$ into RNA in high salt after initiation in low salt

(a) Chromatin was isolated and its template activity determined as detailed in the Materials and Methods section. Each point was assayed with $22\mu\text{g}$ of *E. coli* RNA polymerase fraction 4. (b) Chromatin ($2\mu\text{g}$) was incubated with 4 units of purified *E. coli* K 12 RNA polymerase. Initiation was for 15 min. Propagation was started at zero time by the addition of $(\text{NH}_4)_2\text{SO}_4$, CTP and MgCl_2 , and incorporation of $[^3\text{H}]\text{UTP}$ was monitored by trichloroacetic acid precipitation of samples ($100\mu\text{l}$) (see the Materials and Methods section). The results are expressed as pmol of UTP incorporated/total assay mixture (0.7ml). Incorporation in the absence of template or RNA polymerase was less than 2pmol/assay tube . This background has been subtracted from the data shown. ▲, Fresh medium + 10% serum; △, fresh medium + 0.3% serum.

Determination of initiation sites by sucrose-gradient analysis

The results given above could indicate that in stimulated chromatin there is an increase in the number of binding sites for *E. coli* RNA polymerase. However, this is only true if the length of growing RNA chains is the same for both control and stimulated chromatins. The size of the RNA products synthesized by these two chromatins was analysed by sucrose-gradient centrifugation (Fig. 2). The gradient from control chromatin was indistinguishable from the one of stimulated chromatin. Total nucleotides incorporated were calculated as described above, and the results are given in Table 2. After 35 min of chain elongation the lengths of the RNA chains from both chromatin samples were similar. The number-average size for these RNA molecules was 656 and 648 nucleotides with stimulated and control chromatin respectively. These results are similar to that reported by Cedar & Felsenfeld (1973) of 630 nucleotides for sheared chromatin. Since the sizes of the RNA chains

synthesized by stimulated or control chromatin are essentially the same, the data in Table 1 can be explained by an increase in the number of binding sites for *E. coli* RNA polymerase. One can calculate, on the basis of chain length and of pmol of nucleotides incorporated, that the number of initiation sites in stimulated chromatin is 36% higher than in unstimulated chromatin (see column 4, Table 2). It should be re-emphasized that in both cases the results are independent of enzyme concentration (Table 1).

Determination of initiation sites by using $[\gamma\text{-}^{32}\text{P}]\text{GTP}$

The incorporation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ has been used to measure the number of RNA chains initiated by RNA polymerase (Maitra & Hurwitz, 1965). Since it is known that RNA chains begin with one of these two triphosphates, Cedar & Felsenfeld (1973) used this technique to determine the number of initiation sites in calf thymus chromatin and found results similar to those obtained with initiation in low salt

and propagation in high salt. However, Cedar & Felsenfeld (1973) also showed that $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ incorporation could not be determined accurately with chromatin as template, because $^{32}\text{P}\text{ATP}$ is the substrate for protein kinases that phosphorylate histones (Jergil & Dixon, 1970). We therefore omitted a measurement of the number of initiation sites by using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and simply compared the extent of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ incorporation into RNA by either stimulated or control chromatin. No appreciable incorporation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ into acid-insoluble

material occurs when the *E. coli* RNA polymerase is omitted from the incubation mixture (not shown). The results in the last column of Table 2 show that there is a 40% increase in incorporation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ by chromatin isolated from WI-38 cells 1h after stimulation.

Titration of RNA polymerase with chromatin

In this method for determining the number of initiation sites in chromatin, a fixed concentration of RNA polymerase was titrated with various amounts of template. The initiation period was 15min and

Fig. 2. Sucrose gradient analysis of the RNA products made by WI-38 chromatin

Assay conditions were the same as described in Fig. 1(b). RNA was made by using 2 μg of chromatin and 4 units of enzyme. After 35 min of propagation in high salt, 0.2 ml of the assay mixture was subjected to sucrose gradient analysis as described in the Materials and Methods section (ii). The data are displayed by plotting the percentage of total counts as a function of the fraction number. Total counts on this gradient were 3000 c.p.m. The rRNA and tRNA markers are indicated by arrows.

Fig. 3. Titration of RNA polymerase with chromatin

Increasing amounts of chromatin were incubated with 0.8 unit of *E. coli* K12 RNA polymerase. Incubation conditions were the same as described in the legend to Fig. 1(b). After 35 min of propagation in high salt, samples (100 μl) were assayed for incorporation of $[\text{}^3\text{H}]\text{UTP}$ into trichloroacetic acid-precipitable material. The data are expressed as pmol of UTP incorporated in 35 min. Incorporation of UTP in the absence of template was less than 5 pmol. ▲, Fresh medium + 10% serum; Δ, fresh medium + 0.3% serum.

Table 2. Determination of the number of growing RNA chains by sucrose-gradient analysis and, in part, by incorporation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ into chain termini

Assay conditions were as described in the Materials and Methods section, paragraphs (i), (ii) and (iii) under 'Determination of binding sites for *E. coli* RNA polymerase'. Total nucleotides incorporated were calculated from the data in Fig. 1(b), as described in the text. The average length of RNA chains synthesized was determined from 0.2 ml of each assay tube by sucrose-gradient analysis, as described in paragraph (ii), 'Sucrose-gradient analysis'. Each tube contained 2 μg of chromatin and 4 units of RNA polymerase. Results are the means of five determinations, $\pm$ s.d. Incorporation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was determined as described in paragraph (iii), 'Incorporation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ '.

Template	Nucleotides incorporated (pmol)	Chain length (nucleotides)	Initiations (pmol)	Incorporation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (pmol)
Control chromatin	31 ± 3.7	648 ± 42	0.048	0.042 ± 0.003
Stimulated	43 ± 4.5	656 ± 61	0.066	0.059 ± 0.002

Fig. 4(a). Chromatin template activity of confluent monolayers of WI-38 cells 1 h after a change of medium and (b) incorporation of $[^3\text{H}]\text{UTP}$ into RNA in high salt after initiation in low salt

Chromatin was isolated and its template activity determined as detailed in the Materials and Methods section. Conditions are as described in the legend to Fig. 1(b). ●, Unstimulated controls, no change of medium; Δ, fresh medium+0.3% serum; ○, addition of 10% (w/v) serum to old medium; ▲, fresh medium+10% (w/v) serum.

chain elongation, which was started by addition of high salt, MgCl_2 and CTP, was allowed to continue for 35 min before the reaction was stopped with trichloroacetic acid, as described in the Materials and Methods section. The final extent of $[^3\text{H}]\text{UTP}$ incorporation was used as an indication of the number of initiated RNA chains in each incubation mixture. The number of initiations increases with increasing amounts of chromatin until a plateau is reached, which represents the point at which all RNA polymerase molecules are bound to the template. Fig. 3 shows that this titration point is reached with about 40 and 56 μg of DNA as chromatin from control and stimulated cells respectively. These data again suggest that chromatin from cells stimulated to proliferate has an increased number of initiation sites. It should be noted that the titration curves for both types of chromatin reach approximately the same plateau value when the template is in excess. This reflects the fact that the number of growing chains on excess of template is determined only by the number of bound RNA polymerase molecules, which is identical for both templates in the plateau region.

Effect of changes of medium on chromatin template activity and on the incorporation of $[^3\text{H}]\text{UTP}$ into RNA in high salt after initiation in low salt

The apparent relationship between the increase in chromatin template activity and the number of available initiation sites in chromatin, illustrated in Fig. 1, was investigated in more detail. Rovera & Baserga (1973) have demonstrated a correlation between the extent of increase in chromatin template activity and the extent of cellular proliferation induced in confluent WI-38 fibroblasts. Therefore we measured both chromatin template activity and the number of binding sites available for RNA polymerase in chromatin isolated from WI-38 cells stimulated to proliferate to different degrees by various nutritional changes. Fig. 4(a) shows the template activity of chromatin isolated from confluent monolayers of WI-38 fibroblasts 1 h after nutritional changes. Fresh medium containing 10% (w/v) serum, the addition of 10% serum to the old and, to a much lesser extent, fresh medium containing 0.3% (w/v) serum, caused an increase in chromatin template activity over that of chromatin isolated from cells grown without a

change of medium. The extent of increase in chromatin template activity is proportional to the extent of stimulation of cellular proliferation, as reported by Rovera & Baserga (1973). Fig. 4(b) shows the kinetics of incorporation of [^3H]UTP into RNA in 0.4M-(NH_4) $_2\text{SO}_4$ after initiation in low salt, with the same four chromatin preparations as template. Both the rate and extent of [^3H]UTP incorporation differ for each chromatin sample and are similar to those observed for chromatin template activity (Fig. 4a): thus the addition of fresh medium containing either 10 or 0.3% serum results in 58 and 16% increases, respectively, in [^3H]UTP incorporation over the value obtained for unstimulated cells without changes of medium.

Discussion

The methods described in the present paper are based on the work of Hyman & Davidson (1970) and Cedar & Felsenfeld (1973). The latter authors, using calf thymus DNA and calf thymus chromatin, provided evidence that (1) in the absence of CTP and MgCl_2 , and in low salt, *E. coli* RNA polymerase initiates RNA synthesis on either template; (2) after addition of CTP and MgCl_2 , and in high salt [0.4M-(NH_4) $_2\text{SO}_4$] elongation of initiated RNA chains occurs without reinitiation and (3) the number of initiation sites thus determined is essentially similar to the number of initiation sites determined by the incorporation of γ -labelled nucleoside triphosphates into chain termini. We have confirmed the results of Hyman & Davidson (1970) and Cedar & Felsenfeld (1973) and showed that unsheared chromatin can also be used in a reproducible way to determine the number of binding sites for *E. coli* RNA polymerase. It is not surprising that sheared chromatin has a higher number of binding sites for *E. coli* RNA polymerase than unsheared chromatin, since it is known that sheared chromatin has a higher template activity for RNA synthesis than unsheared chromatin (Couch & Anderson, 1973).

Our results also show that chromatin isolated from WI-38 cells 1 h after stimulation to proliferate by nutritional changes has a higher number of binding sites for *E. coli* RNA polymerase than chromatin isolated from unstimulated confluent WI-38 cells. The number of initiation sites appears to be increased in chromatin of stimulated cells whether the number of binding sites is measured by the technique of propagation in high salt after initiation in low salt, or by measuring the incorporation of [γ - ^{32}P]GTP into chain termini (Maitra & Hurwitz, 1965). The extent of variation in these experiments never exceeded 10% of the mean and most of the times it was in the order of 5%. Figs. 1 and 4 give a good idea of the reproducibility of the results in experiments carried out several weeks apart.

The increase in number of binding sites of chromatin from stimulated cells parallels the increase in chromatin template activity that has also been found in WI-38 human diploid fibroblasts stimulated to proliferate by a change in medium. This is particularly interesting because an increase in chromatin template activity has been reported early in the pre-replicative phase of several models of stimulated DNA synthesis, including the oestrogen-stimulated uterus (Barker & Warren, 1966; Teng & Hamilton, 1968), rat liver cells stimulated to divide by partial hepatectomy (Thaler & Vilee, 1967; Bannai & Terayama, 1969; Mayfield & Bonner, 1972), phytohaemagglutinin-stimulated lymphocytes (Hirschhorn *et al.*, 1969), isoproterenol-stimulated salivary glands (Novi & Baserga, 1972), and the ventral prostate of castrated rats after administration of androgens (Couch & Anderson, 1973). In this respect it should be noted that our results are in agreement with those of Cox *et al.* (1973), who showed that oestradiol increases by two to three times the number of elongating RNA chains in chick oviduct chromatin. The method used by Cox *et al.* (1973) to determine the number of growing RNA chains was different from those used in the present experiments, but the results obtained are in agreement, showing that appropriate stimulation of cell proliferation causes an increase in the number of initiation sites. This, of course, does not yet establish whether new RNA species are actually synthesized by chromatin of stimulated cells. However, our data and those of Cox *et al.* (1973) suggest that most, if not all, of the increase in chromatin template activity found in models of stimulated DNA synthesis may be due to an increase in the number of initiation sites, rather than to an increase in the rate of elongation of growing RNA chains.

There remains to discuss the use in these experiments of *E. coli* RNA polymerase, which has been severely criticized as a tool for probing chromatin function, especially by Butterworth *et al.* (1971), Keshgegian & Furth (1972) and Tsai & Saunders (1973). However, apart from several other reports indicating that *E. coli* RNA polymerase has a considerable degree of fidelity in transcribing eukaryotic chromatin (Gilmour & Paul, 1970; Axel *et al.*, 1973), this objection probably does not apply to the present case since, as mentioned above, it has been shown that the increase in chromatin template activity occurring in WI-38 human diploid fibroblasts stimulated to proliferate by a change of medium can be detected whether one uses *E. coli* RNA polymerase or the homologous polymerase (Farber *et al.*, 1972).

In conclusion our results indicate that when confluent monolayers of WI-38 human diploid fibroblasts are stimulated to proliferate by nutritional changes, there is within 1 h after stimulation an increase in the number of chromatin binding sites for *E. coli* RNA polymerase. This change parallels that in chromatin

template activity and probably reflects an increase in the number of initiation sites in the chromatin of stimulated cells.

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