Mechanism of Stimulation of Chromatin Transcription by Putrescine: Effects on Rate of Elongation and Number of Initiation Sites Utilized†

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ABSTRACT: As a part of a study on the mechanisms of stimulation of liver chromatin transcription by polyamines we examined the effect of putrescine on the rate of elongation of RNA chains and the number of initiation sites utilized. Techniques which permitted the separation of the binding-initiation phase from the chain elongation stage were used. Putrescine did not change the number of sites used by *E. coli* RNA polymerase (ribonucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) for transcription on chromatin or DNA. The diamine caused a 40–50% stimulation of the rate of elongation per RNA chain on both chromatin and DNA. The presence of putrescine during the elongation phase of transcription promoted the synthesis of longer chains on chromatin but not on DNA. Putrescine stimulates the overall transcription of liver chromatin and at concentrations above 20 mM prevents the formation of a plateau in incorporation. The degree of stimulation of transcription produced by putrescine depended on the enzyme/template ratio. Release of the plateau by putrescine was partially inhibited by rifampicin and was accompanied by the synthesis of longer RNA chains and an increase in the frequency of initiation and/or reinitiation. This work suggests that putrescine may facilitate transcription on chromatin by promoting higher rates of synthesis and a more efficient use of initiation sites without causing alterations in the number of sites utilized.

Studies on cell systems undergoing growth, such as regenerating rat liver, indicate a correlation between the timing of RNA and polyamine synthesis (Dykstra and Herbst, 1965; Fausto, 1969; Raina et al., 1966; Russell and Lombardini, 1971). Although it is possible that polyamines have a regulatory function in RNA metabolism during growth processes in eukaryotic systems (Fausto et al., 1975; Raina and Jänne, 1975), no direct evidence for this role has been provided by studies conducted in vivo (Fillingame and Morris, 1973). If the polyamines participate in the control of RNA transcription at the early stages of liver regeneration, only putrescine would be likely to fulfill this function because a marked increase in putrescine occurs early after partial hepatectomy while changes in spermidine and spermine are not detected until many hours later (Jänne, 1967). In addition, the conversion of spermidine to putrescine is markedly enhanced in regenerating liver (Hölttä et al., 1973). Thus, it is of considerable interest to determine if and by what mechanisms putrescine may stimulate RNA synthesis on chromatin in vitro.

Spermidine and spermine have been reported to affect several different stages of RNA synthesis on a DNA template: rate of polymerization of RNA chains (Richardson et al., 1966); length of RNA chains (Jänne et al., 1975); specificity of initiation (Gumport and Weiss, 1969); and dissociation of the polymerase from DNA (Nuss and Herbst, 1975). Information on the possible effects of putrescine on DNA transcription is limited to the observation that it stimulates the overall rate of ribonucleotide incorporation (Jänne et al., 1975; Krakow, 1963). Very few studies have been reported on polyamine effects on chromatin transcription (Jänne et al., 1975; Moruzzi et al., 1975) and there are conflicting results regarding the action of polyamines on the number of sites on the template used for initiation of RNA synthesis. The interpretation of most of these results is difficult because the techniques utilized do not permit a clear separation between the initiation and elongation phases of transcription. Moreover, at the optimal concentrations of magnesium required for transcription, spermidine and spermine often cause precipitation of chromatin. To avoid such difficulties, we have employed techniques (Cedar and Felsenfeld, 1973; Tsai et al., 1975) which enabled us to study separately the effects of putrescine on initiation and elongation. Such studies are important for the understanding of the biological effects of polyamines. If putrescine increases the number of initiation sites on liver chromatin, it could exert a selective effect on gene transcription during growth and regeneration.

Materials and Methods

Materials

Nucleoside triphosphates were obtained from P-L Biochemicals. [5,6-^{3}H]UTP (35–50 Ci/mmol), [γ-^{32}P]ATP (2–10 Ci/mmol), [γ-^{32}P]GTP (2–10 Ci/mmol), hyamine hydroxide, and Omnifluor were purchased from New England Nuclear Corp. Rifampicin was purchased from Sigma Chemical Co. and putrescine and spermidine were supplied by Ames Laboratories. *E. coli* K-12 RNA polymerase was purchased from commercial suppliers (Miles Laboratory, grade I, specific activity 231–583 units/mg; grade II, specific activity 1063 units/mg; Sigma Chemical Co., specific activity 673 units/mg) or was prepared from *E. coli* K-12 cells (¾ log phase, Grain Processing Corp.), by the method of Burgess (1969), and purified through two glycerol gradients. The estimated specific activity of this preparation was 800–900 units/mg. The phosphocellulose column step was omitted in each of these enzyme preparations.

All rats used in these experiments were male albino rats, Holtzman strain, from Charles River Breeding Lab., weighing 120 to 200 g. The animals were given food and water ad libitum and maintained in temperature-controlled rooms under alternating 12-h light–dark cycles.

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0006-2960/78/0417-0102$01.00/0

Methods

Isolation of Nuclei for Preparation of Chromatin. Nuclei were prepared by a modification of the procedure of Bucher and Swaffield (1965). Rats were killed by decapitation. Livers were rapidly excised and placed in cold homogenization buffer (0.25 M sucrose, 0.05 M Tris, pH 7.6, 0.003 M $CaCl_2$). All subsequent procedures were carried out at 4 °C. Livers were weighed, minced, and and homogenized in 5 vol (by liver weight) of homogenization buffer in a Potter-Elvehjem homogenizer. The homogenate was filtered through two layers of gauze and centrifuged at 600*g* for 10 min. The crude nuclear pellet was resuspended gently (using a specially fitted glass bulb) in 6 vol of a solution containing 0.25 M sucrose, 0.0001 M $MgCl_2$, 0.1% Tween-80, and then homogenized with 10 strokes in a tight-fitting Dounce homogenizer. The suspension was centrifuged at 600*g* for 10 min. The pellet was resuspended with a glass bulb in 3.5 vol of 0.25 M sucrose, 0.0006 M Tris, pH 8.0, and centrifuged at 600*g* for 10 min. Resuspension in 0.25 M sucrose, 0.006 M Tris, pH 8.0, was repeated once, followed by centrifugation at 2000*g* for 10 min.

Preparation of Chromatin. Chromatin was prepared from purified nuclei by a modification of the procedure of Huang and Huang (1969). The saline–EDTA step was omitted. The final nuclear pellet was resuspended in 5 vol of 0.05 M Tris, pH 8.0, with a loose-fitting Dounce homogenizer and centrifuged at 10 000*g* for 10 min. Resuspension in 0.05 M Tris, pH 8.0, and centrifugation were repeated once. Resuspension was performed twice in 5 vol of 0.01 M Tris, pH 8.0, followed by centrifugation each time at 10 000*g* for 15 min. The resuspension step was then repeated twice in 5 vol of 0.005 M Tris, pH 8.0, followed by centrifugation each time at 15 000*g* for 15 min. The final gelatinous pellet was resuspended in 0.005 M Tris, pH 8.0, to a DNA concentration of 0.4–0.5 mg/mL. The chromatin to be used in unsheared form was shaken gently on a rotary shaker for 4–5 h at 4 °C to disperse any remaining aggregates. For sheared chromatin, the resuspended gelatinous pellet was sheared on ice for 90 s in a VirTis homogenizer at a speed of 4000–50 000 rpm. The sheared sample was then shaken for 40 min at 4 °C and centrifuged at 10 000*g* for 30 min. The supernatant was removed and used in this form as sheared chromatin.

Biochemical Analysis of Chromatin. The basic procedure of Munro and Fleck (1966) was used to determine the DNA, RNA, and protein content of chromatin. RNA content was estimated by the orcinol method on supernatants obtained after hydrolysis in 0.3 N KOH at 37 °C. DNA content was determined by the diphenylamine method on supernatants obtained after incubation in 1.0 N perchloric acid at 70 °C. Protein content was estimated by the method of Lowry et al. (1951) on the remaining pellet after incubation in 1.0 N NaOH at 90 °C. DNA, RNA, and protein standards were run simultaneously with chromatin samples in the KOH–perchloric acid procedure to estimate degree of loss. Recovery of all three components was generally 80–95% by this method. The amount of chromatin used in transcription assays was estimated from the diphenylamine results and is expressed as μg of DNA in chromatin.

Preparation of Rat Liver DNA for in Vitro Incubations. DNA was prepared from purified rat liver nuclei according to the procedure of Marmur (1969). Nuclei were prepared in the same manner as for chromatin preparations, except that 2% citric acid was included in the Tween-80 buffer. The DNA was stored at −70 °C. DNA concentration was determined by the diphenylamine method, using calf thymus DNA as a standard.

Standard Assay Mixture for Transcription in Vitro. The incubation conditions of Bonner et al. (1968) were used with slight modifications. The reaction mixture consisted of 40 mM Tris, pH 7.9, 12 mM β-mercaptoethanol, 1 mM $MnCl_2$, 0.24 mM each ATP, GTP, and CTP, 0.12 mM [^{3}H]UTP, 10 μg of chromatin (or DNA), and 2 units of *E. coli* RNA polymerase in a volume of 0.25 mL. Incubations were performed at 37 °C in a shaking water bath. This set of conditions is referred to as the standard, low ionic strength assay mixture. Any modifications of these conditions are noted for each experiment.

Activity of Endogenous Rat Liver RNA Polymerase. Incorporation of labeled UTP into RNA by chromatin-bound endogenous rat liver RNA polymerase was determined for each set of assay conditions used in the experiments presented in this paper. Endogenous activity of chromatin was assayed in the absence of added *E. coli* RNA polymerase, and this value was subtracted from total incorporation to give *E. coli* RNA polymerase-dependent incorporation. With the standard assay mixture, endogenous incorporation amounted to less than 10% of total incorporation (endogenous incorporation plus *E. coli* RNA polymerase dependent incorporation).

Preincubation–Elongation Procedure. A modification of the methods of Cedar and Felsenfeld (1973) and Tsai et al. (1975) was used when initiation and elongation were to be examined separately. In the preincubation step, DNA or chromatin and *E. coli* RNA polymerase were incubated for 5 to 15 min at 37 °C in 0.25 mL of preincubation buffer containing 40 mM Tris, pH 7.9, 12 mM β-mercaptoethanol, 1 mM $MnCl_2$, 4 mM $MgCl_2$, 0.24 mM each ATP and GTP. At the end of the preincubation period, elongation was started by the addition of CTP to 0.24 mM [^{3}H]UTP to 0.12 mM and 1 or 4 μg of rifampicin. Total time of elongation was 20 min for chromatin and 60 min for DNA. Modifications of these conditions are noted for each experiment. Endogenous incorporation was determined for each assay and the results are expressed as *E. coli* RNA polymerase-dependent incorporation.

Determination of Acid-Precipitable Radioactivity. Incorporation of [^{3}H]UTP into acid-precipitable material was determined after trichloroacetic acid precipitation and filtration into Whatman GF/C filters. Incorporation of [γ-^{32}P]ATP and [γ-^{32}P]GTP was estimated after precipitation with perchloric acid, according to the procedure of Maitra and Hurwitz (1965). This technique was essential for obtaining low blank values when using radioactive nucleotides of high specific activity.

Results

Unsheared chromatin preparation had protein–DNA ratios of 2.22 and RNA–DNA ratios of 0.062. The absorbance ratios of the chromatin preparations were: 260/230 = 0.92; 260/240 = 1.29; 260/280 = 1.52; and 320/260 = 0.071. Unsheared chromatin was less soluble than the sheared samples and had a higher protein content as indicated by higher 320/260 ratios.

When the activities of sheared and unsheared chromatin prepared from the same batch of nuclei were compared under identical assay conditions, the sheared sample was found to be 1.5–2.5 times more active than the unsheared sample. The template activity of chromatin was approximately 7% of that of purified rat liver DNA (Figure 1) and the template saturation curves (using a fixed amount of enzyme) were nearly identical with those published by Bonner et al. (1968). If liver chromatin is stored at 4 °C at a DNA concentration of 0.4–0.5 mg of DNA/mL, the template activity increases by approxi-

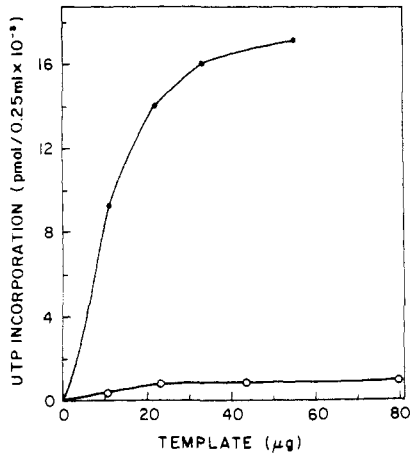

FIGURE 1: Transcriptional activities of sheared chromatin and rat liver DNA. Ten units of *E. coli* RNA polymerase were incubated with the amounts of sheared chromatin (—○—) or DNA (—●—) indicated on the abscissa. The incubations were for 10 min at 37 °C in the standard assay mixture containing 40 mM Tris, pH 7.9, 12 mM β-mercaptoethanol, 1 mM $MnCl_2$, 4 mM $MgCl_2$, 0.24 mM each ATP, GTP, and CTP and 0.12 mM [^{3}H]UTP (100 dpm/pmol). UTP incorporation into acid-insoluble material was determined after precipitation of the incubation mixtures with trichloroacetic acid followed by filtration onto Whatman GF/C filters. UTP incorporation is expressed as pmol of nucleotide incorporated per 0.25 mL of incubation mixture × 10^{-3}.

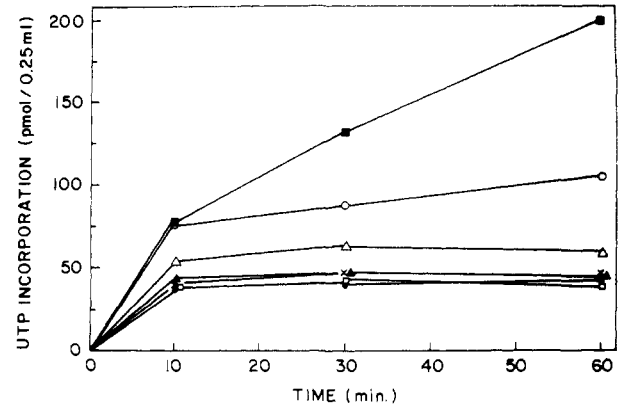

FIGURE 2: Effect of various concentrations of putrescine on transcription of chromatin. Ten micrograms of unsheared liver chromatin and 2 units of *E. coli* RNA polymerase were incubated at 37 °C in the standard assay mixture using [^{3}H]UTP (150 dpm/pmol) as the labeled substrate. Putrescine was added to the incubation mixture in the following concentrations: no putrescine (—●—); 1 mM (—□—); 2.5 mM (—X—); 5 mM (—▲—); 10 mM (—△—); 20 mM (—○—); 40 mM (—■—). Samples were removed from the incubation mixtures at the times indicated on the abscissa. The ordinate shows the amount of [^{3}H]UTP incorporated into acid-precipitable material expressed as pmol of UTP incorporated per 0.25 mL of incubation mixture.

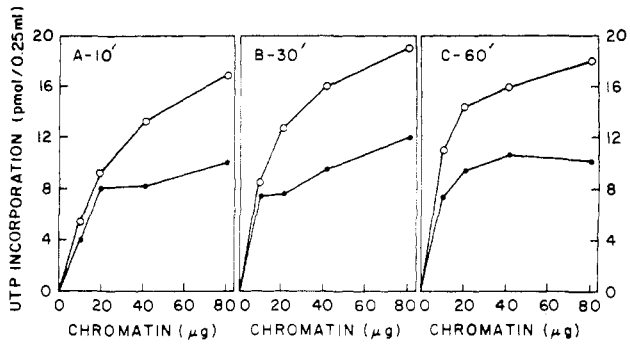

FIGURE 3: Dependence of putrescine effect on enzyme/template ratio. Ten units of *E. coli* RNA polymerase was incubated with the amounts of sheared chromatin indicated on the abscissa in the standard assay mixture (see Methods and legend to Figure 1) in the absence (—●—) or presence (—○—) of 20 mM putrescine. Aliquots were removed at 10 min (panel A), 30 min (panel B), and 60 min (panel C). The abscissa shows the amount of chromatin included in the assay mixture. The ordinate shows the amount of [^{3}H]UTP incorporated into acid-precipitable material expressed as pmol of UTP incorporated per 0.25 mL of incubation mixture.

mately 10% per day for at least 5 days. Preparations stored in a more concentrated form (0.7–1.0 mg DNA/mL) show better stability.

To determine what proportion of the acid-precipitable radioactivity was incorporated into RNA, two separate assays using [^{3}H]UTP or [γ-^{32}P]GTP + [γ-^{32}P]ATP as substrates in the standard incubation mixture were used. Approximately 99% and 90% of the labeled product were digested by ribonuclease in assays using ^{3}H or ^{32}P nucleotides, respectively. Proteinase K digested approximately 2% of the tritiated product and 10–15% of the ^{32}P-labeled material. For nucleotide analysis of the labeled transcription product, unsheared chromatin was incubated in the standard assay mixture using [γ-^{32}P]GTP + [γ-^{32}P]ATP as labeled substrates. After acid precipitation and alkaline hydrolysis (Bremer et al., 1965) the neutralized material was analyzed by thin-layer chromatography on poly(ethylenimine)-cellulose (Randerath and Randerath, 1967). Both pppGp and pppAp were identified by two-dimensional chromatography (Gallant et al., 1976). More than 89% of the radioactivity migrated with these two nucleotides.

Effect of Various Concentrations of Putrescine on Chromatin Transcription. In order to determine the best conditions for studying the effects of putrescine on chromatin transcription, the kinetics of RNA synthesis was examined in the presence of various concentrations of putrescine. It can be seen in Figure 2 that with the standard, low ionic strength assay mixture containing unsheared chromatin, net incorporation of UTP ceases after 10 min in the absence of putrescine. This cessation of incorporation has been termed plateau formation (Fuchs et al., 1967; Morris and Gould, 1971; Nuss and Herbst, 1975) and occurs somewhat later (30 min) on sheared chromatin. Ten, 20, and 40 mM putrescine stimulate incorporation during the initial phase of incubation (zero time to 10 min), but only 20 and 40 mM putrescine are effective in preventing formation of the plateau.

Standard assay mixtures (see Methods) containing 10 units of *E. coli* RNA polymerase were incubated for 10, 30, and 60 min with varying amounts of sheared chromatin. One series of incubation mixtures contained no putrescine while 20 mM putrescine was included in the other series. The results are shown in Figure 3 after correction for endogenous incorporation. The degree of stimulation by putrescine depends both on the enzyme/template ratio and the time of incubation. With amounts of chromatin above 40 μg there is a 50–80% stimulation of incorporation at all incubation times. With chromatin amounts ranging from 10 to 20 μg only a small stimulation is seen at 10 min of incubation. The data shown on panel A of Figure 3 (10-min incubation period) as well as the results of an identical experiment using DNA were used to calculate K_m and V_{max} values on Eadie–Hofstee plots (Table I). The V_{max} values indicate that putrescine stimulates the overall rate of transcription on both templates. The K_m is approximately 2.5-fold higher when putrescine is included in the incubation mixture. If these apparent K_m values are used as estimates of the number of binding sites for enzyme on the template, the results would indicate the following: (1) there are fewer sites for enzyme on either template when putrescine is present; (2)

TABLE I: Apparent K_m and V_{max} Values for Chromatin and DNA Transcription with and without Putrescine.[a]

Assay conditions	App K_m	K_m(+Put)/ K_m(−Put)	V_{max}	V_{max}(+Put)/ V_{max}(−Put)
Chromatin	35.7		1500	
		1.17		1.75
Chromatin + putrescine	41.9		2620	
D NA	16.0		23200	
		2.57		2.11
DNA + putrescine	41.2		49000	

[a] Apparent K_m and V_{max} values have been derived by Eadie-Hofstee plots from the curves shown in Figure 3. V_{max} values are determined from the y-intercept, and apparent K_m values from the ratio y intercept/x intercept.

DNA has only twice as many binding sites per μg of template as chromatin. These conclusions are inconsistent with data obtained with more precise methods to examine the binding-initiation phase separately (see below). We conclude that the estimation of K_m values in this overall transcription assay does not provide accurate estimates of the number of binding sites for RNA polymerase on the template.

A preliminary experiment was performed (a) to determine whether putrescine added after the plateau stage had formed could renew incorporation, and (b) whether such an effect could be prevented by rifampicin. Addition of 40 mM putrescine 10 min after the plateau has formed promotes a renewal of incorporation. This renewal is partially sensitive to rifampicin, and the incorporation reaches a new plateau level 5 min after the addition of putrescine plus the drug. No further incorporation was seen when 8.5 μg of extra template was added at 10 min so it is assumed that all RNA polymerase molecules are bound to the original template at the plateau state. Rifampicin when added at the start of the reaction completely prevented initiation. This experiment suggests that the rifampicin-resistant renewal of synthesis in the presence of putrescine represents further elongation of previously initiated RNA chains and that the rifampicin-sensitive synthesis represents additional round(s) of initiation by the enzyme. The formation of a plateau on chromatin appears to be due at least in part to a block in further elongation of RNA chains and to a failure of RNA polymerase to undergo further initiation and/or reinitiations.

Elongation of RNA Chains and Extent of Initiation and Reinitiation at the Plateau Stage in Presence of Putrescine. Since over 95% of RNA chains synthesized in vitro on chromatin begin with ATP or GTP (Keshegian et al., 1973), incorporation of [γ-^{32}P]ATP and [γ-^{32}P]GTP can be used to estimate number of initiations or number of RNA chains and the [^{3}H]UTP/[γ-^{32}P]ATP + [γ-^{32}P]GTP ratios can be used to estimate chain length (Maitra and Hurwitz, 1965). This type of analysis was applied to incubations performed in the presence and absence of putrescine, as shown in Table II. Incubation mixtures lacking *E. coli* RNA polymerase or chromatin were monitored for enzyme- or template-independent incorporation of ^{32}P.

The results presented in Table II indicate that: (a) the rate of initiation of new chains is sharply reduced after the formation of a plateau in the absence of putrescine; the ratio between number of chains at 50 min and number of chains at 10 min is 1.11 for incubation mixtures lacking putrescine (sample A, Table II); (b) the addition of putrescine causes the synthesis of a significant number of new chains (2.36-fold increase between 10 and 50 min of incubation, sample C in Table II). The efficiency of initiation is quite low (<25%) indicating that a significant number of RNA polymerase molecules do not initiate an RNA chain on chromatin under these conditions.

The sizes of the chains present in standard incubation mixtures and in mixtures which contain putrescine + rifampicin or putrescine alone are also shown in Table II. The average size of RNA chains remains constant after plateau formation has occurred but the size of chains increased by 55% in B (putrescine + rifampicin) and 20% in C (putrescine alone). The smaller size increase in the mixtures containing putrescine alone (mixture C, Table II) can be explained by the presence of a greater number of newly initiated chains (since rifampicin was not added) which would decrease the ratio of [^{3}H]UTP to [γ-^{32}P]ATP + [γ-^{32}P]GTP incorporated.

These experiments (using mixtures A, B, and C) were repeated under identical conditions but using [^{3}H]UTP as the

TABLE II: Effect of Putrescine on the Number and Size of RNA Chains Transcribed at the Plateau Stage.[a]

	Additions at plateau	Time (min)	Chains (pmol)	Chain no. at 50 min/ chain no. at 10 min	UTP incorp. (pmol)	Nucleotides per chain (s value)	Efficiency of initiation
(A)	None	10	0.42		24.9	202 (6.2 S)	0.12
		50	0.47	1.11	26.5	193 (6.1 S)	0.13
(B)	Putrescine +	10	0.41		23.4	197 (6.1 S)	0.12
	rifampicin	50	0.41	0.99	37.2	315 (7.6 S)	0.12
(C)	Putrescine	10	0.38		23.1	208 (6.3 S)	0.11
		50	0.90	2.36	62.8	239 (6.7 S)	0.26

[a] Two different assays were performed on the following mixtures: Three identical mixtures, A, B, and C, containing 10 μg of unsheared chromatin and 2 units of *E. coli* RNA polymerase were incubated for 10 min in the standard assay mixture (see legend to Figure 1). At 10 min, 40 mM putrescine (final concentration) plus 1 μg of rifampicin were added to B, and 40 mM putrescine (final concentration) was added to C, and the reactions were continued for an additional 40 min. In the first assay, 0.08 mM [γ-^{32}P]ATP, 0.08 mM [γ-^{32}P]GTP (800 cpm/pmol each), 0.08 mM CTP, and 0.08 mM unlabeled UTP were used. Aliquots were removed at 10 and 50 min of incubation. In the second assay, 0.08 mM each ATP, GTP, and CTP and 0.08 mM [^{3}H]UTP (105 dpm/pmol) were used. Aliquots were removed at 10 and 50 min of incubation. Aliquots containing ^{32}P label were processed as described in Materials and Methods. Chain number was calculated from the incorporation of ^{32}P into acid-precipitable radioactivity, after correction for incorporation in the absence of template or *E. coli* RNA polymerase (the blank values varied from 0.04 to 0.07 pmol incorporated during the period of the assay). Chain length was calculated from the relative amount of [^{3}H]UTP and [γ-^{32}P]ATP + [γ-^{32}P]GTP incorporation. Efficiency of initiation was calculated from the ratio of number of RNA chains synthesized to the number of *E. coli* RNA polymerase molecules bound to the template, according to the procedure of Tsai et al. (1975). All polymerase molecules present in the assay mixtures were assumed to be bound to the template.

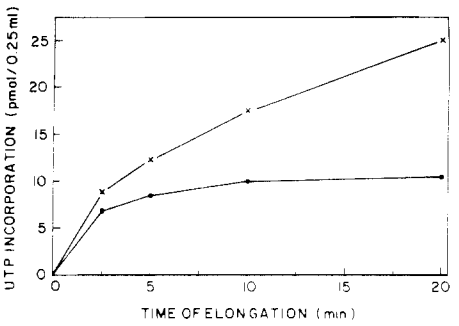

FIGURE 4: Kinetics of elongation in the presence and absence of rifampicin. Thirty micrograms of unsheared rat liver chromatin and 1 unit of *E. coli* RNA polymerase were preincubated for 15 min in 40 mM Tris, pH 7.9, 12 mM β-mercaptoethanol, 1 mM $MnCl_2$, 4 mM $MgCl_2$, 0.24 mM each ATP and GTP. Elongation was started by the addition of [^{3}H]UTP (400 dpm/pmol) to 0.12 mM, CTP to 0.24 mM, and putrescine to 40 mM. No rifampicin (—X—); 1 μg of rifampicin added at the start of elongation (—●—). Aliquots were removed at the times of elongation indicated on the abscissa. The ordinate shows the amount of [^{3}H]UTP incorporated into acid-precipitable material expressed as pmol of UTP incorporated per 0.25 mL of incubation mixture. The radioactivity in acid-insoluble material was determined after precipitation of the incubation mixtures with trichloroacetic acid followed by filtration onto Whatman GF/C filters.

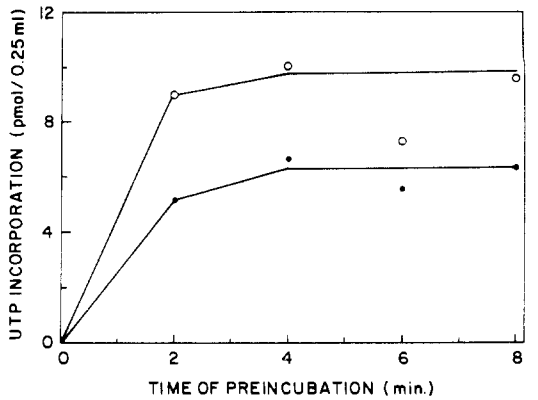

FIGURE 5: Time required for completion of binding. A series of mixtures containing 5 μg of unsheared rat liver chromatin and 1 unit of *E. coli* RNA polymerase was preincubated (see Methods and legend to Figure 3) for the indicated times in the absence (—●—) or presence (—○—) of 20 mM putrescine. Elongation was started by the addition of [^{3}H]UTP to 0.12 mM (400 dpm/pmol), CTP to 0.24 mM, and 1 μg of rifampicin. Reactions were stopped after 20 min of elongation. The abscissa shows the length of the preincubation period. The ordinate shows the amount of [^{3}H]UTP incorporated into acid-precipitable material expressed as pmol of UTP incorporated per 0.25 mL of incubation mixture.

labeled precursor and the RNA was isolated and analyzed in sucrose gradients (Hyman and Davidson, 1970; Cedar and Felsenfeld, 1973; Groner et al., 1975). The following results were obtained: (a) plateau formation in the absence of putrescine was accompanied by an inhibition in the frequency of initiation of new RNA chains (16% increase in number of chains between 10 and 30 min); and (b) addition of putrescine at the plateau stage promoted the synthesis of a significant number of new chains by *E. coli* RNA polymerase (2.34-fold increase between 10 and 30 min). This is in agreement with the results obtained with the isotope ratio method.

Preincubation-Elongation Procedure for the Analysis of Chromatin Transcription. In order to study the effects of putrescine on the initiation and elongation phases of transcription (before a plateau stage is reached), it was necessary to use techniques which (a) separate the initiation from the elongation phase; (b) allow no more than one initiation per site so that the number of initiations (RNA chains) could be equated with the number of sites used for synthesis; (c) permit only one round of synthesis to occur, without reinitiation. During preincubation in a low ionic strength assay medium containing only two nucleotides (ATP and GTP), *E. coli* RNA polymerase binds to the template and forms rifampicin-resistant preinitiation complexes at initiation sites (Pribnow, 1975). Formation of the first nucleotide bond and elongation are started by the addition of CTP, and UTP in the presence of rifampicin. Elongation is allowed to proceed to completion of incorporation. Under these conditions, rifampicin prevents (1) initiation by RNA polymerase not associated with the template in preinitiation complexes; (2) multiple initiations at a single initiation site; (3) reinitiation at the end of a single round of synthesis.

Pilot experiments showed that: (1) when ATP and GTP were omitted from the preincubation mixture and all four nucleotides were added with rifampicin, the final level of synthesis was found to be 10% higher than when ATP and GTP were included at the preincubation stage: (2) the incorporation of [^{3}H]UTP which occurred during the preincubation phase in the presence of ATP, GTP, *and* [^{3}H]UTP (CTP was added at the end of preincubation) amounted to less than 9% of the total incorporation at the end of the elongation phase. These results indicate that the preincubation conditions used for the following experiments do not allow significant levels of incorporation to occur during the preincubation and permit an adequate separation of the initiation and elongation phases.

Unsheared chromatin and *E. coli* RNA polymerase were preincubated for 5 min (see Methods, preincubation-elongation procedure). RNA synthesis was then started by the addition of [^{3}H]UTP, CTP, and 20 mM putrescine in the presence or absence of rifampicin. The results of this assay are shown in Figure 4. In the absence of rifampicin, incorporation is linear for at least 20 min, whereas, in the presence of rifampicin, incorporation stops after 10 min. These results indicate that 1 μg of rifampicin is effective in preventing reinitiation on chromatin and that elongation in the presence of rifampicin is essentially complete by 10 min. The standard elongation time used in the following experiments on chromatin was 20 min. To determine the time necessary for complete binding of enzyme to template during preincubation, chromatin and *E. coli* RNA polymerase were preincubated for various times in the presence or absence of putrescine. Total incorporation was determined at the end of 20 min of elongation (Figure 5). Binding during the preincubation period is relatively rapid and is essentially complete by 4 min. No significant difference was observed between preincubation performed in the presence or absence of putrescine (samples taken between 2 and 8 min of preincubation). A 15-min preincubation time was used for all of the following experiments.

Effect of Putrescine on the Number of Sites Utilized for Initiation and on the Role of Elongation per RNA Chain on Chromatin. In the following experiments, we have used incorporation of [γ-^{32}P]ATP and [γ-^{32}P]GTP to estimate chain number (number of initiation sites) and the ratio of [^{3}H]UTP to [γ-^{32}P]ATP + [γ-^{32}P]GTP incorporation to estimate the rate of elongation per RNA chain.

(a) Number of RNA Chains. Three preincubation mixtures, A, B, and C, contained 5 μg of unsheared chromatin, 1 unit of *E. coli* polymerase, and [γ-^{32}P]ATP and [γ-^{32}P]GTP. Elongation was started by the addition of UTP, CTP, and rifampicin and continued for 20 min. Mixture A contained no putrescine for preincubation or elongation; mixture B contained 20 mM putrescine for elongation only; mixture C contained 20 mM putrescine for preincubation and elongation. Incuba-

TABLE III: Effect of Putrescine on Initiation[a] and Elongation[b] on Chromatin.

	Putrescine		pmol of chains initiated	Time of elong	[^{3}H]UTP incorp		Rate of elong (nuc per min	% stimulation by	Nucleotides per chain
	preinc	elong	(γ-[^{32}P] incorp)	(min)	pmol UTP incorp.	pmol of nucleotides	per chain)	putrescine	(s value)
(A)	−	−	0.05	2.5	2.2	7.4	58		
				5.0	2.3	8.0	32		
				10.0	3.1	8.7			
				20.0	3.2	11.0			217 (6.4 S)
(B)	−	+	0.06	2.5	3.1	10.6	83	43	
				5.0	3.9	13.6	51	59	
				10.0	4.7	16.1			
				20.0	4.9	16.6			303 (7.5 S)
(C)	+	+	0.05	2.5	3.4	11.9	88	50	
				5.0	3.9	13.6	50	50	
				10.0	4.9	16.9			
				20.0	5.2	18.0			332 (7.8 S)

[a] Three assay mixtures (A, B, and C) containing 5 μg of unsheared chromatin and 1 unit of *E. coli* RNA polymerase were preincubated for 15 min in a medium containing 40 mM Tris, pH 7.9, 12 mM β-mercaptoethanol, 1 mM $MnCl_2$, 4 mM $MgCl_2$, and 0.08 mM each of [γ-^{32}P]ATP and [γ-^{32}P]GTP. Elongation was started by the addition of UTP and CTP to 0.08 mM each and 1 μg of rifampicin. Mixture A contained no putrescine for preincubation or elongation; in B putrescine was present only during elongation and was added simultaneously with UTP, CTP, and rifampicin; in C, putrescine was present in the preincubation and elongation phases. Incorporation of ^{32}P-labeled nucleotides into acid-precipitable material was determined at the end of 20 min of elongation by the method described by Maitra and Hurwitz (1965). The amount of chains initiated was calculated from the total amount of UTP incorporated × 3.45 (average base composition). This number was divided by the average number of nucleotides per RNA chain synthesized. All RNA chains are assumed to be labeled equally. [b] The same assay mixtures were used but the preincubation mixture contained 0.08 mM each of ATP and GTP but no labeled nucleotide. [^{3}H]UTP (400 dpm/pmol) and CTP were added at the start of elongation together with 1 μg of rifampicin. Samples were removed at 2.5, 5, 10, and 20 min of elongation for determination of acid-precipitable radioactivity. The rate of elongation was determined from the number of RNA chains and the amount of [^{3}H]UTP incorporation at 2.5 and 5 min of elongation. The number of nucleotides per chain was calculated from the chain number and the amount of [^{3}H]UTP incorporated after 20 min of elongation. Values have been corrected for incorporation of labeled nucleotides in the absence of template or *E. coli* RNA polymerase.

tion mixtures lacking RNA polymerase or chromatin were used to monitor template-independent ^{32}P incorporation. The results of this experiment are presented in Table III. No significant difference in the number of RNA chains synthesized in the three different assay mixtures was found. In mixture C, putrescine was present in the preincubation step when binding and initiation of chains occur. We conclude that putrescine (at a concentration known to stimulate the overall rate of RNA synthesis) has no effect on the number of sites used for initiation.

(b) Rate of Elongation per RNA Chain. The same mixtures A, B, and C were used but unlabeled ATP and GTP were present during the preincubation and [^{3}H]UTP was added at the start of the elongation period (Table III). Samples of the incubation mixture were removed at 2.5, 5, 10, and 20 min of elongation. Rates of elongation were determined between 2.5 and 5 min of elongation when net incorporation of ^{3}H is taking place; the number and length of RNA chains were determined at 20 min. The rate of elongation was calculated as nucleotides per min per chain based on the total amount of [^{3}H]UTP incorporated per unit of time and the total number of chains synthesized. The results presented in Table III indicate that putrescine present only during the elongation phase (mixture B) stimulates the rate of elongation per RNA chain by 43% at 2.5 min and 59% at 5 min. Comparable stimulations occurred when putrescine was present during the preincubation and elongation phases of the assay (mixture C). The final level of UTP incorporation (determined 20 min after the start of elongation) is higher in the presence of putrescine (mixtures B and C). Calculation of the size of RNA chains at 20 min indicates that the average size of RNA chains elongated in the presence of putrescine is 40–50% larger than chains synthesized in absence of the diamine.

Effect of Putrescine on the Number of Sites Used for Initiation and on the Rate of Elongation per RNA Chain on DNA. For comparison to the results obtained on chromatin, experiments were performed on rat liver DNA to test the effect of putrescine on the rate of elongation per RNA chain and on the number of sites used for initiation. Preliminary assays were performed to determine the conditions to be used with DNA as a template. It was found that 4 μg of rifampicin was more effective than 1 μg in producing kinetics of elongation similar to those seen with chromatin as a template. Incubations for 60 min were necessary to permit the completion of elongation on DNA. Essentially the same set of assays presented in Table III for chromatin was performed for DNA transcription. The results are presented in Table IV.

(a) Number of RNA Chains. The results indicate that putrescine has no effect on the number of RNA chains initiated on DNA under these asay conditions. Approximately ten times more chains are synthesized on DNA than on chromatin, which is in agreement with results reported by others (Cedar and Felsenfeld, 1973).

(b) Rate of Elongation. Putrescine included in the incubation mixture produced a 49–57% stimulation of the rate of elongation per RNA chain, nearly identical with the stimulation seen on chromatin. The absolute rate of elongation on DNA is twofold greater than the rate of elongation on chromatin. Putrescine did not increase the size of RNA chains synthesized on DNA, in contrast with results on chromatin. Nuss and Herbst (1975) have also reported that spermidine stimulates the transcription of T4 DNA without altering the size of the RNA chains.

As shown in Tables III and IV the rate of elongation at 2.5 and 5 min is approximately 58 and 32 nucleotides per min per chain, respectively, for chromatin. For DNA the rates of

TABLE IV: Effect of Putrescine on Initiation and Elongation on DNA.[a]

	Putrescine		pmol of chains initiated ([^{32}P] incorp)	Time of elong (min)	[^{3}H]UTP incorp		Rate of elong (nuc per min per chain)	% stimulation by putrescine	Nucleotide per chain (*s* value)
	preinc	elong			pmol of UTP incorp.	pmol of nucleotides			
(A)	−	−	0.54	2.5	51.3	177	131		
				5.0	68.3	236	87		
				30.0	94.3	325			
				60.0	99.4	343			633 (10.7 S)
(B)	−	+	0.53	2.5	73.9	255	194	49	
				5.0	89.4	308	8	35	
				30.0	96.3	332			
				60.0	101.4	350			666 (10.9 S)
(C)	+	+	0.55	2.5	80.1	276	196	50	
				5.0	85.7	296	105	21	
				30.0	94.2	325			
				60.0	97.4	336			607 (10.4 S)

[a] Assay conditions are the same as that described in the legend to Table III with the following modifications: 5 μg of DNA was used instead of chromatin; 4 μg of rifampicin was used instead of 1 μg. The elongation time was extended to 60 min.

elongation are 131 and 87 nucleotides per min per chain at 2.5 and 5 min, respectively. These values are in close agreement with those reported by Cedar and Felsenfeld (1973) with calf thymus chromatin and DNA.

Discussion

We have studied the effect of putrescine on the rate of elongation per RNA chain and the number of sites utilized for initiation during chromatin transcription. The mechanisms by which putrescine reverses the plateau phenomenon were also analyzed.

Although bacterial RNA polymerase is not the homologous enzyme, the use of *E. coli* RNA polymerase in this work has definite advantages. It is the most thoroughly studied RNA polymerase (bacterial or eukaryotic), and its mechanism of action and requirements for synthesis during different phases of transcription are better characterized than for any other RNA polymerase. On the basis of previous evidence, *E. coli* RNA polymerase seems to be a useful probe for general template properties of chromatin (Hill and Baserga, 1974; Hwang et al., 1974) and for transcription of specific DNA sequences in chromatin (Astrin, 1973; Axel et al., 1973; Tsai et al., 1976). The sites occupied by the bacterial enzyme are not necessarily the same as those which would be used by the homologous rat liver RNA polymerase in vitro. Both competition (Tsai et al., 1976) and lack of competition (Cedar, 1975) have been observed between bacterial and mammalian RNA polymerase for the same initiation regions. In the former case, the results strongly suggest that the main difference between the two enzymes is the efficiency of transcription of certain DNA regions and not the type of initiation regions selected. Several additional observations on deficiencies in initiation by certain eukaryotic RNA polymerase preparations and the possible need for additional enzyme factors by eukaryotic polymerase (Chuang and Chuang, 1975; Howk et al., 1974; Lee and Dahmus, 1973; Mandel and Chambon, 1974) suggest that the bacterial enzyme is a reasonable choice for the present experiments and is not necessarily less reliable than the homologous rat liver enzyme.

Plateau formation in our rat liver chromatin transcription system under low ionic strength assay conditions is accompanied by a block in further elongation of at least some RNA chains, a decrease in the frequency of initiation, and a failure to release bound RNA chains effectively from polymerase-template-RNA complexes. The addition of putrescine causes a renewal of synthesis by promoting further extensions of RNA chains and new rounds of chain initiation. It is possible that, at the plateau stage, further elongation is necessary before release of RNA chains and new initiations can take place. Since putrescine does not increase the number of initiation sites utilized on chromatin, the new rounds of initiation following addition of putrescine at the plateau stage may represent more effective use of initiation sites, that is, additional synthesis on the same sites utilized prior to plateau formation. Due to the low efficiency of initiation, we cannot determine whether these additional rounds of synthesis are actually the result of recycling and reinitiation by *E. coli* RNA polymerase or the result of initiations by bound, but previously unutilized polymerase molecules. Although endogenous RNase activity is not a factor in formation of a plateau or in release of the plateau by putrescine (data not shown), we cannot exclude an effect of this enzyme on the length of the RNA chain synthesized.

An essential aspect of the preincubation-elongation technique is the assumption that no more than one initiation is permitted per site, so that the number of chains synthesized can be equated with the number of sites utilized. Two observations support this assumption: (1) a close correlation was found between the number of initiation sites on T7 DNA estimated by the preincubation technique and other methods; (2) studies of promoter regions and recycling of σ factor (Ruger, 1975; Wu et al., 1975) make it unlikely that more than one initiation could occur at a particular site under the preincubation conditions used in our experiments. The type of initiation detected is likely to occur specifically on native DNA regions and not on denatured or nicked regions. This is supported by the fact that *E. coli* holoenzyme (used in our experiments) does not initiate to any significant extent on single-stranded or nicked regions in chromatin (Cedar, 1975; Chuang and Chuang, 1975; Tsai et al., 1975). The RNA polymerase bound at nicked sites is also very rapidly inactivated by rifampicin, which was included in our assays.

Using the preincubation-elongation technique, we found that putrescine does not change the number of sites on chromatin or DNA utilized for initiation. Although we did not examine in detail the kinetics of binding, some experiments suggest that there may be no significant difference in the time required to complete the binding step in the presence and absence of putrescine. These results do not support the proposal

that putrescine, by a direct interaction with the template, alters the affinity or availability of RNA polymerase for true initiation sites as has been suggested for spermine and spermidine (Abraham, 1968; Moruzzi et al., 1975). Our experiments do not rule out the possibility that putrescine may redistribute initiation sites without changing the total number of sites used.

In summary, these studies indicate that putrescine stimulates transcription on chromatin in vitro by *E. coli* RNA polymerase by promoting a faster rate of elongation per RNA chain as well as the synthesis of RNA chains of greater average chain after the initial round of site selection has taken place. The evidence argues against an effect of putrescine on the specificity of initiation, at least with respect to the number of sites utilized. Molecular hybridization analysis of gene expression at the early stages of hepatic regeneration suggests that liver hypertrophy may occur without massive derepression of previously uncopied genes (Colbert et al., 1977). Thus, a possible role of putrescine in cell growth could be that of facilitating transcription by promoting more rapid rates of synthesis and more efficient use of initiation sites.

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