Biochem. J. (1975) **146**, 697–703
Printed in Great Britain

The Effect of Spermine on Transcription of Mammalian Chromatin by Mammalian Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase

By GIOVANNI MORUZZI, BRUNO BARBIROLI, MARIA STELLA MORUZZI and BRUNA TADOLINI
Istituto di Chimica Biologica dell'Università di Bologna, Via Irnerio 48, 40126 Bologna, Italy

(*Received 5 August* 1974)

Isolated rat liver nuclei demonstrate an increased ability to synthesize RNA in the presence of either spermine or spermidine. Spermidine has more effect on the low-salt α-amanitin-insensitive reaction, and spermine has more effect on the high-salt α-amanitin-sensitive reaction. Spermine is effective at concentrations of 0.1 mM and 1 μM, showing a biphasic effect. The RNA polymerase activity associated with nuclear chromatin is increased in the presence of spermine only at a concentration of 0.1 mM. Also the transcription of deproteinized liver DNA by liver form-B polymerase or *Escherichia coli* enzyme is more efficient in the presence of 0.1 mM-spermine. Only when liver chromatin is transcribed by its homologous enzyme (and not by *E. coli* enzyme) is spermine active at both 0.1 mM and 1 μM as in purified nuclei. The lower concentration of spermine (1 μM) is able to affect chromatin transcription by increasing the affinity of chromatin for the enzyme. Our findings suggest a regulatory role of spermine at the level of genome transcription.

Studies on the physiological role of the aliphatic polyamines, spermine and spermidine, have increased almost exponentially in the last few years, and have implicated them in a number of different processes [see reviews by Cohen (1971) and Bachrach (1973)].

High concentrations of the aliphatic polyamines repeatedly observed in animal tissues undergoing growth and division (Cohen, 1971; Bachrach, 1973), together with their presence in relatively high concentrations in vegetable embryos (Moruzzi & Caldarera, 1964), first suggested a possible relationship between these polycations and nucleic acids. However, a few reports are in disagreement (Fillingame & Morris, 1973; Kay & Pegg, 1973). Much research done under different experimental conditions has shown an even closer relationship between polyamines and RNA synthesis. For example, studies *in vitro* on rat liver nucleoli (Russell *et al.*, 1971) and purified prostate-gland nuclei (Caldarera *et al.*, 1968; Pegg *et al.*, 1970), and on more simplified systems (Abraham, 1968; Stirpe & Novello, 1970), have demonstrated a stimulation of DNA-dependent RNA polymerase activity by polyamines.

Reports by Herbst *et al.* (1973) and Moruzzi *et al.* (1974) have implicated spermine in the mechanism of regulation of gene expression, even if the molecular mechanism by which spermine and spermidine act is still unknown.

To gain some insight into the possible role of polyamines in the regulation of RNA synthesis we have studied the transcription of rat liver chromatin in the presence of spermine. Three aspects have been particularly investigated. First, we have studied the effect of spermine and spermidine on the ability of purified nuclei to synthesize RNA over a wide range of concentrations. Secondly, the effect of spermine on the activity of RNA polymerase molecules associated with chromatin and finally the effect of this polyamine on the template activity of liver chromatin were studied.

Materials and Methods

All experiments were performed on 7-week-old male albino rats of the Wistar strain maintained since weaning on the controlled feeding schedules developed by Potter *et al.* (1968) and described in a previous paper (Barbiroli *et al.*, 1975).

The purification of liver nuclei, chromatin and DNA preparations, extraction and separation of liver form-A RNA polymerase and form-B RNA polymerase, the assays of enzyme activities and the release of proteins from chromatin were performed as described previously (Barbiroli *et al.*, 1975).

Spermine or spermidine were added to the incubation mixtures at the final concentrations indicated in the separate experiments.

Results

Effects of spermine or spermidine on nuclear RNA synthesis in vitro

Before studying in more detail the single components involved in transcription we measured the ability of liver nuclei, isolated from adult rats maintained on controlled feeding schedules, to synthesize RNA *in vitro* in the presence of either spermine or spermidine.

All experiments reported in this paper were performed at 09:00h, a time of day at which a low rate of RNA synthesis has been found (Barbiroli *et al.*, 1973) in the liver of rats maintained under our experimental conditions.

Experiments reported in Fig. 1 indicate that polyamines selectively stimulate the two types of RNA polymerase activities. In low concentrations of salt (form-A polymerase) there is a peak of stimulation of α-amanitin-insensitive activity (180% over the control values) at about 4mM-spermidine (Fig. 1*a*, dashed line), whereas spermine shows very limited stimulation (+30%) and at a lower concentration (0.5mM) (see Fig. 1*a*, solid line). On the other hand, when RNA synthesis is measured in high concentrations of salt, stimulation (80–90% over the control values) of α-amanitin-sensitive RNA polymerase activities is found at two different spermine concentrations (0.1mM and 1 μM) (see Fig. 1*b*, solid line).

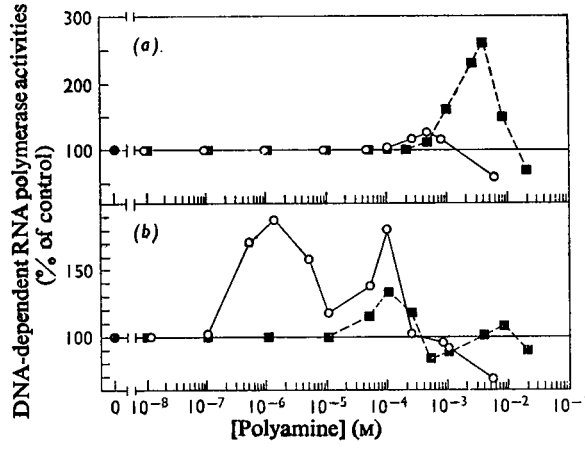

Fig. 1. *Effect of spermine and spermidine on the ability of rat liver nuclei to synthesize RNA in vitro in the presence or absence of α-amanitin*

Nuclei were isolated from the liver of rats killed at 09:00h. Samples of nuclear suspension, containing 90–100 μg of DNA, were assayed for their ability to incorporate [^{14}C]ATP as described by Barbiroli *et al.* (1975), in the presence or absence of either spermine (○) or spermidine (■). Radioactivity incorporated into acid-insoluble material was then estimated. Results are reported as percentage of control, the control values being 132pmol of [^{14}C]ATP/mg of nuclear DNA (low-salt α-amanitin-insensitive activity) (*a*) and 736pmol of [^{14}C]ATP/mg of nuclear DNA (high-salt α-amanitin-sensitive activity) (*b*).

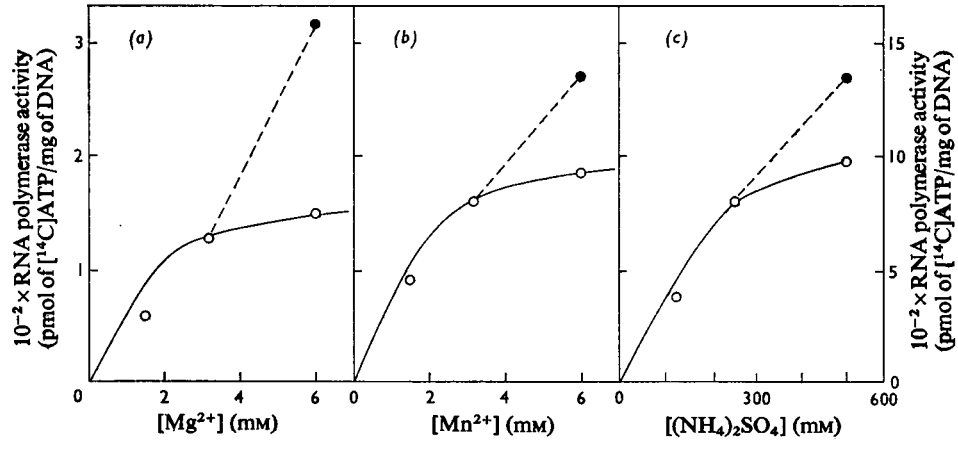

Fig. 2. *Effect of spermine, spermidine, Mg^{2+}, Mn^{2+} and $(NH_4)_2SO_4$ on the ability of isolated rat liver nuclei to synthesize RNA in vitro in the presence or absence of α-amanitin*

Portions of the nuclear suspension obtained as described by Barbiroli *et al.* (1975), containing 90–100 μg of DNA, were assayed for their ability to incorporate [^{14}C]ATP as described by Barbiroli *et al.* (1975). Radioactivity incorporated into acid-insoluble material was then measured and expressed per mg of nuclear DNA. Spermidine (final concentration 4mM) was added to the low-salt reaction mixture in the presence of α-amanitin (*a*) when assayed in the presence of 3.3mM-$MgCl_2$. Spermine (final concentration 1 μM) was added to the high-salt reaction mixture when assayed in the presence of 3.3mM-$MnCl_2$ (*b*) or 250 mM-$(NH_4)_2SO_4$ (*c*). (*a*) Low-salt reaction; (*b*) and (*c*) high-salt reaction.

Spermidine addition results in a smaller stimulating effect at 0.1 mM with no stimulation occurring at all the other concentrations tested (Fig. 1*b*, dashed line).

Inhibition of form-B RNA polymerase activities has also been found at concentrations over 1 mM-spermine or -spermidine, whereas form-A enzyme (α-amanitin-insensitive) is inhibited by spermidine concentrations over 10 mM and by spermine over 1 mM.

Results reported in Fig. 2 indicate that increased concentrations of Mg^{2+}, Mn^{2+} or $(NH_4)_2SO_4$ over the corresponding optimum values are unable to stimulate further RNA synthesis measured *in vitro* in whole nuclei, whereas the specific polyamine does.

Effect of spermine on RNA polymerase activity associated with chromatin

It has been reported that the elongation step of RNA synthesis appears to be independent of the ionic strength up to rather high values both in *Escherichia coli* (Fuchs *et al.*, 1967) and mammals (Butterworth *et al.*, 1971). Therefore we have assayed the chromatin-associated RNA polymerase activities in high salt concentrations (300 mM) in order to have a measure of the influence of spermine on the elongation step during RNA synthesis. In fact polymerase molecules bound to chromatin do not re-initiate in high salt concentrations (Barbiroli *et al.*, 1975).

Addition of spermine at optimum salt concentration (300 mM) results in a further stimulation of total endogenous activities only at about 0.1 mM (95% over the control values) (Fig. 3, open circles). Form-B enzyme is more sensitive to the presence of spermine, but a smaller stimulation is found for form-A enzyme.

The salt dependence of chromatin-associated RNA polymerase activity could be correlated to the solubility of chromatin itself and to the removal of protein from chromatin at high salt concentration. We have measured the influence of the most effective concentration of spermine (0.1 mM) on the removal of protein by $(NH_4)_2SO_4$ and the results are reported in Table 1. Spermine at the highest stimulatory concentration does not significantly remove any more proteins than does $(NH_4)_2SO_4$.

Fig. 4 reports the kinetics of endogenous RNA synthesis in the presence or absence of the most effective dose of spermine (0.1 mM) at 300 mM-$(NH_4)_2SO_4$. We have investigated whether the differences found in the presence or absence of spermine could be regarded as gross differences in

Table 1. *Effect of spermine on the release of proteins by $(NH_4)_2SO_4$*

Chromatin samples containing 1 mg of DNA and 1.58 mg of protein were tested with various concentrations of $(NH_4)_2SO_4$ in the presence or absence of 0.1 mM-spermine; the removal of protein was measured as described by Barbiroli *et al.* (1975). Results represent the average ±S.E.M. of six to eight determinations performed on different occasions.

$[(NH_4)_2SO_4]$ (mM)	Protein removed (% of total)	
	Spermine absent	+0.1 mM-Spermine
180	20±1.7	21±1.3
240	22±2.0	23±1.8
300	25±1.8	23±1.6

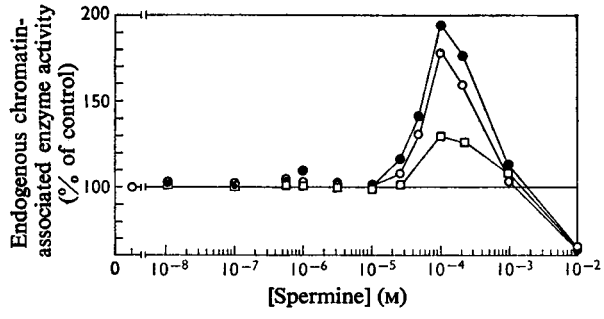

Fig. 3. *Effect of spermine on liver chromatin-associated RNA polymerase activity*

Chromatin (containing 40–50 μg of DNA) was assayed for the ability to incorporate [^{3}H]UTP into RNA in the presence of several concentrations of spermine. Enzyme activity was assayed in 300 mM-$(NH_4)_2SO_4$ and as indicated in Fig. 2 and the preceding paper. Results are reported as percentages of control values. ○, Total activity (control value being 16 pmol of [^{3}H]UTP/100 μg of DNA); ●, α-amanitin-sensitive activity (control value being 11 pmol of [^{3}H]UTP/100 μg of DNA); □, α-amanitin-insensitive activity (control value being 5 pmol of [^{3}H]UTP/100 μg of DNA).

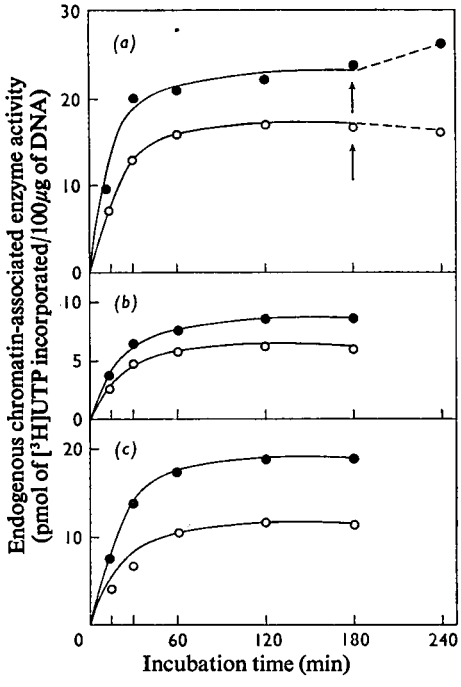

Fig. 4. *Comparison of time kinetics of liver chromatin-associated polymerase activity in the presence or absence of spermine*

Reaction mixtures were incubated at 37°C up to 4h, and contained 40–50μg of DNA as chromatin in a final concentration of 300mM-$(NH_4)_2SO_4$. ○, Control; ●, +spermine (0.1mM). After 3h (arrows), portions (10μl) of 86mM-unlabelled UTP were added and the reaction was followed for another hour. (*a*) Total activity; (*b*) α-amanitin-insensitive activity; (*c*) α-amanitin-sensitive activity.

RNAase* activity. In fact after 1h of incubation, radioactive-isotope incorporation into RNA reached a plateau. This behaviour could be due to a cessation of RNA synthesis or to an equilibrium between RNA synthesis and degradation. A 50-fold dilution of [^{3}H]UTP precursor in the incubation mixture after 3h of incubation does not result in a decrease in radioactivity in the acid-insoluble product.

Effect of spermine on liver form-B or E. coli RNA polymerases

In these experiments we have evaluated the effect of spermine on liver form-B RNA polymerase with rat liver DNA as template. Results in Fig. 5 show that the polyamine is able to stimulate RNA synthesis

* Abbreviation: RNAase, ribonuclease.

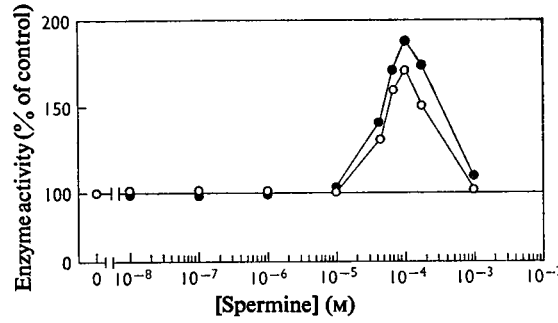

Fig. 5. *Effect of spermine on transcription of liver deproteinized DNA by liver form-B RNA polymerase or E. coli enzyme*

Twenty enzyme units of liver form-B RNA polymerase or 0.1 enzyme unit of *E. coli* RNA polymerase were incubated with 20μg of deproteinized liver DNA as described by Barbiroli *et al.* (1975), in the presence or absence of spermine. Reactions were carried out in 100mM-$(NH_4)_2SO_4$. ○, *E. coli* enzyme; ●, liver form-B enzyme. Results are expressed as percentages of control. Control values were 75pmol/10min for *E. coli* enzyme and 16pmol/10min for form-B liver enzyme.

catalysed by the liver enzyme only at a concentration of about 0.1mM, being insensitive at higher or lower concentrations. We have also studied the effect of spermine when *E. coli* enzyme is used to transcribe rat liver DNA instead of liver enzyme. We established a pattern of spermine stimulation very similar to that obtained with the liver enzyme (Fig. 5).

Effect of spermine on the efficiency of liver chromatin to act as template for liver form-B or E. coli RNA polymerase

An optimum $(NH_4)_2SO_4$ concentration of between 180 and 220mM is required by form-B enzyme to transcribe hen oviduct or rat liver chromatins (Butterworth *et al.*, 1971; Cox *et al.*, 1973). However, other results (Barbiroli *et al.*, 1975) have shown a lower optimum (<0.1M) when both enzyme and chromatin are extracted from the liver of rats starved for 16h in the re-feeding regimen of Potter *et al.* (1968), i.e. our experimental conditions. All the experiments dealing with form-B enzyme and chromatin were performed with a salt concentration of 100mM.

Spermine is able to stimulate chromatin transcription by form-B RNA polymerase (open symbols, Fig. 6) at two different concentrations (0.1mM and 1μM) by 105 and 92% over the corresponding control values respectively.

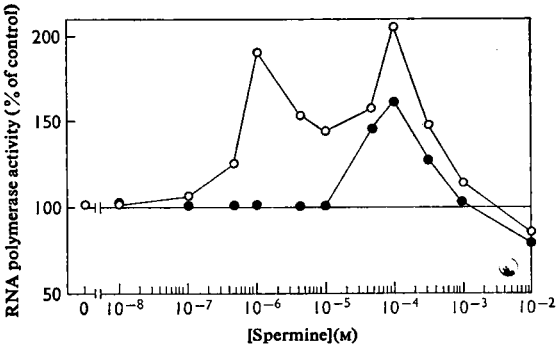

Fig. 6. *Effect of spermine on transcription of liver chromatin by liver form-B RNA polymerase or E. coli enzyme*

Twenty enzyme units of liver form-B RNA polymerase or 0.1 enzyme unit of *E. coli* RNA polymerase were incubated with 40–50 μg of chromatin DNA as described by Barbiroli *et al.* (1975), in the presence or absence of spermine. Reactions were carried out in 100 mM-$(NH_4)_2SO_4$. ●, *E. coli* enzyme; ○, liver form-B enzyme. Results are expressed as percentage of control. Control values were 36 pmol/30 min for *E. coli* enzyme, and 7 pmol/30 min for liver form-B enzyme. To determine RNA synthesis due only to exogenous enzyme, radioactive isotope incorporated in assays containing no enzyme was subtracted for each concentration of spermine tested.

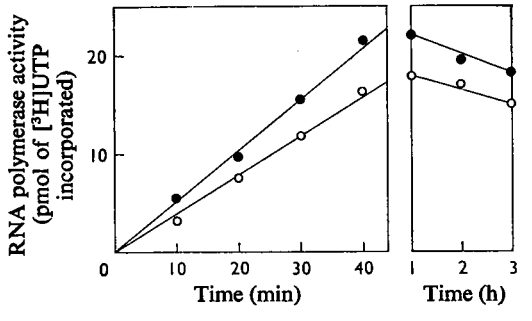

Fig. 7. *Effect of spermine on the ability of liver chromatin to act as template for liver form-B RNA polymerase*

Liver form-B RNA polymerase (20 units) was assayed in 100 mM-$(NH_4)_2SO_4$ with chromatin (80–100 μg of DNA) as template. To determine RNA synthesis due only to exogenous enzyme, radioactive-isotope incorporation in assays containing no enzyme was subtracted for each time-point. ○, Control; ●, +1 μM-spermine.

On the other hand, when *E. coli* enzyme is used to transcribe liver chromatin the presence of spermine in the incubation mixture results in a smaller stimulating effect and only at a concentration of about 0.1 mM (+65%). Also this experiment was performed in 100 mM-$(NH_4)_2SO_4$.

As reported in Fig. 7, the activity of exogenous form-B RNA polymerase is approx. 40% higher when 1 μM-spermine is added to the incubation mixture. This experiment was performed under conditions in which chromatin template was in excess. Higher stimulations (+90%) with a similar pattern (experiments not reported) are obtained when chromatin is limiting. Therefore it appears that it is not necessary to saturate the chromatin DNA with exogenous enzyme to observe differences in template efficiency, as reported by Cox *et al.* (1973). After 40 min in either the presence or the absence of spermine the reaction is no longer linear and longer incubation times show a progressive decline of label from RNA without significant differences between the two conditions studied (±1 μM-spermine). It seems reasonable to assume [see results of Cox *et al.* (1973) and Cox (1973)] that this behaviour is due to RNAases contaminating the enzyme preparation.

We have also evaluated the amount of chromatin required to saturate a fixed amount of enzyme in the presence or absence of 1 μM-spermine (Fig. 8*a*). Twenty enzyme units are more readily saturated by chromatin in the presence of 1 μM-spermine than in the control experiments. Double-reciprocal plots (Fig. 8*b*) give very similar theoretical maximum velocities in the absence or presence of spermine (21.7 and 24.0 pmol of UTP/15 min respectively); the apparent K_m value is largely decreased by spermine: 55.6 μM-chromatin DNA in the control reaction and 32.1 μM-chromatin DNA when 1 μM-spermine is present in the reaction mixture.

Discussion

In the presence of several concentrations of either spermine or spermidine in the incubation mixture, isolated rat liver nuclei demonstrate an increased ability to synthesize RNA. Spermidine is more effective on the low-salt α-amanitin-insensitive RNA polymerase reaction, whereas the presence of spermine in the incubation mixture results in a large stimulation of the high-salt α-amanitin-sensitive RNA polymerase reaction. Previous reports from this (Caldarera *et al.*, 1968; Moruzzi *et al.*, 1971) and other laboratories (Abraham, 1968; Stirpe & Novello, 1970; Russell *et al.*, 1971) have shown a stimulatory effect of spermine or spermidine on the RNA polymerase reaction measured in purified nuclei or by using solubilized form-B enzyme and DNA as template, but all of them had always considered concentrations of polyamines between 0.1 and 1 mM.

Our present results demonstrate a specific action of spermine and spermidine on nucleoplasmic and nucleolar RNA polymerase reactions respectively, and that the presence of spermine in the incubation

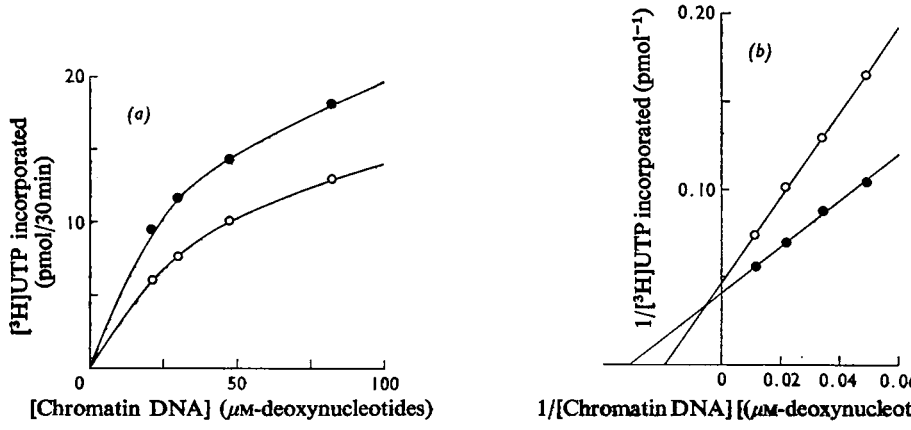

Fig. 8. *Effect of spermine on the ability of liver chromatin to act as template for liver form-B RNA polymerase*

(*a*) Liver form-B polymerase (20 units) was assayed in 100 mM-$(NH_4)_2SO_4$ with various concentrations of liver chromatin as template. To determine RNA synthesis due only to exogenous (added) enzyme, radioactive-isotope incorporation in assays containing no enzyme was subtracted for each concentration tested. Chromatin DNA concentrations are expressed as molarity of deoxynucleotides (average mol.wt. 309). ○, Control; ●, +1 μM-spermine. (*b*) Double-reciprocal plots of (*a*). Apparent K_m values were 53.4 μM-deoxynucleotides (control) or 32.1 μM-deoxynucleotides (in the presence of 1 μM-spermine). Theoretical maximal velocities were 21.7 pmol of [³H]UTP/30 min (control) or 24.0 pmol of [³H]UTP/30 min (+1 μM-spermine).

medium results in a stimulatory effect at two different concentrations (0.1 mM and 1 μM). No straightforward interpretation of the biphasic pattern of spermine stimulation is available, but a similar behaviour has been observed when RNA polymerase activity is measured in the presence of several concentrations of KCl or $(NH_4)_2SO_4$ (Butterworth *et al.*, 1971; Barbiroli *et al.*, 1975) in purified systems.

The inability of higher concentrations of Mn^{2+} ions or $(NH_4)_2SO_4$ over their optimum for the reaction to stimulate RNA synthesis further, together with the peculiar double peak of spermine stimulation of the α-amanitin-sensitive reaction, indicates the possibility of two separate effects of this polycation on RNA synthesis. Therefore we studied this reaction and the single components involved in transcription under the action of spermine in more detail.

First, we studied the chromatin-associated RNA polymerase activities, which represent elongation of RNA chains (Butterworth *et al.*, 1971; Cox *et al.*, 1973). Spermine is able to enhance the endogenous activities of form-A polymerase and form-B polymerase in chromatin, only at a concentration of about 0.1 mM, by 30 and 95% over the control values respectively.

This stimulatory effect is not due to inhibition of RNAase activities present in the chromatin preparations, as dilution of the [³H]UTP pool with unlabelled UTP after 3 h of incubation does not modify the acid-insoluble radioactivity measured 1 h later. Further, spermine stimulation of chromatin-associated RNA polymerase activity is not due to removal of chromatin proteins which might restrict the movement of RNA polymerase molecules.

The experiments on transcription of deproteinized liver DNA by liver form-B RNA polymerase or by *E. coli* enzyme again showed a stimulation of RNA synthesis by spermine only at a concentration of about 0.1 mM.

These results do not establish whether this stimulation is due to an effect on the enzyme molecules, on the DNA template or on the enzyme–template complex. However, spermine stimulation of chromatin transcription by liver form-B enzyme at two different concentrations (1 μM and 0.1 mM), together with its ineffectiveness at 1 μM when *E. coli* enzyme transcribes liver chromatin, suggests a very specific effect of 1 μM-spermine on chromatin template. This possibility is also supported by the non-specific transcription of liver chromatin by bacterial enzymes (Butterworth *et al.*, 1971; Maryanka & Gould, 1973).

Time kinetics of chromatin transcription by liver enzyme in the presence or absence of 1 μM-spermine have shown a significantly increased activity due to spermine. The possibility that this difference was due to inhibition of RNAase activity contaminating the enzyme or the chromatin preparations was excluded by following the reaction for 3 h, after which it is no longer linear. In fact no difference was found in the behaviour in the presence and absence of spermine. Further, 40-fold dilution of the [³H]UTP

with unlabelled UTP after 40min did not result in differences in the presence or absence of spermine (results not reported).

By determining the amount of chromatin needed to saturate a fixed amount of liver enzyme in the presence or absence of 1 μM-spermine one can obtain a comparable estimate of the affinity of chromatin for the enzyme or in turn an estimate of the binding sites for the enzyme on the chromatin. In the absence of spermine the apparent K_m value is 53.4 μM-deoxynucleotides, whereas in the presence of 1 μM-spermine the apparent K_m is decreased to 32 μM-deoxynucleotides. On the contrary, the theoretical maximum velocity in either the presence or the absence of spermine is not significantly changed. This leads us to think that spermine at this concentration acts on the chromatin template and not on the enzyme molecules.

The present results demonstrate two separate effects of spermine, at the genome level. A general effect, apparently non-specific, at about 0.1 mM, was evident in all conditions studied, i.e. purified nuclei, chromatin-associated RNA polymerase activity and DNA and chromatin transcription either by bacterial or liver enzymes. And a second one at about 1 μM which becomes evident only in purified nuclei and when liver chromatin is transcribed by its homologous enzyme, which appears to be very specific. We interpret the modification of the chromatin template as being a protein-mediated effect on the protein–DNA complex to allow the transcription of new or additional RNA products.

Beyond this it would be premature to speculate whether this is achieved by establishing new promotor sites for enzyme binding, by more efficient transcription of existing sites by changes in protein secondary structure, or reorientation of histone and/or non-histone proteins which may act as regulators of gene expression (Paul & Gilmour, 1968; Elgin *et al.*, 1971).

We are grateful to Mr. G. Negroni for his excellent technical assistance. This work was supported by a grant from the Consiglio Nazionale delle Ricerche, Roma, Italy.

References

Abraham, K. A. (1968) *Eur. J. Biochem.* **5**, 143–146
Bachrach, U. (1973) *Function of Naturally Occurring Polyamines*, Academic Press, New York and London
Barbiroli, B., Moruzzi, M. S., Monti, M. G. & Tadolini, B. (1973) *Biochem. Biophys. Res. Commun.* **54**, 62–68
Barbiroli, B., Tadolini, B., Moruzzi, M. S. & Monti, M. G. (1975) *Biochem. J.* **146**, 687–696
Butterworth, P. H. W., Cox, R. F. & Chesterton, C. J. (1971) *Eur. J. Biochem.* **23**, 229–241
Caldarera, C. M., Moruzzi, M. S., Barbiroli, B. & Moruzzi, G. (1968) *Biochem. Biophys. Res. Commun.* **33**, 266–271
Cohen, S. S. (1971) *Introduction to Polyamines*, Prentice–Hall, Englewood Cliffs
Cox, R. F. (1973) *Eur. J. Biochem.* **39**, 49–61
Cox, R. F., Haines, M. E. & Casey, N. H. (1973) *Eur. J. Biochem.* **32**, 513–524
Elgin, S. C. R., Froehner, S. C., Smart, J. C. & Bonner, J. (1971) in *Advances in Cell and Molecular Biology* (Du Praw, E. J., ed.), pp. 1–57, Academic Press, London
Fillingame, R. H. & Morris, D. R. (1973) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **32**, 621
Fuchs, E., Millette, R. L., Zilling, W. & Walter, G. (1967) *Eur. J. Biochem.* **3**, 183–193
Herbst, E. J., Byus, C. V. & Buss, D. L. (1973) in *Polyamines in Normal and Neoplastic Growth* (Russell, D. H., ed.), pp. 71–90, Raven Press, New York
Kay, J. E. & Pegg, A. E. (1973) *FEBS Lett.* **29**, 301–304
Maryanka, D. & Gould, H. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 1161–1165
Moruzzi, G. & Caldarera, C. M. (1964) *Arch. Biochem. Biophys.* **105**, 209–210
Moruzzi, G., Barbiroli, B., Corti, A. & Caldarera, C. M. (1971) *Ital. J. Biochem.* **20**, 6–15
Moruzzi, G., Caldarera, C. M. & Casti, A. (1974) *Mol. Cell. Biochem.* **3**, 153–161
Paul, J. & Gilmour, R. S. (1968) *J. Mol. Biol.* **34**, 305–316
Pegg, A. E., Lockwood, D. H. & Williams-Ashman, H. G. (1970) *Biochem. J.* **117**, 17–31
Potter, V. R., Baril, E. F., Watanabe, M. & Wittle, E. D. (1968) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **27**, 1238–1245
Russell, D. H., Levy, C. C., Taylor, R. L., Gfeller, E. & Sterns, D. N. (1971) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **30**, 1093
Stirpe, F. & Novello, F. (1970) *Eur. J. Biochem.* **15**, 505–512