Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

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Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **57** (1974), n.3-4, p. 285–293.

Accademia Nazionale dei Lincei

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Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Accademia Nazionale dei Lincei, 1974.

Biologia molecolare. — *On the template activity of yeast chromatin* (*). Nota (**) di ERNESTO DI MAURO, NICOLA VIOLA, PATRIZIA ANNESSI e MARIA ARCÀ, presentata dal Socio G. MONTALENTI.

RIASSUNTO. — Vengono riportati i risultati dello studio della capacità trascrittiva della cromatina di *Saccharomyces cerevisiae*. Si dimostra che nelle nostre condizioni sperimentali la cromatina di lievito possiede attività RNA polimerasica endogena, che tale attività è completamente resistente ad α-amanitina ed altamente resistente ad AF/013; ciò indica da un lato l'assenza dalla cromatina della RNA polimerasi II, dall'altro una bassa capacità di riinizio della trascrizione da parte delle altre forme di RNA polimerasi. Si dimostra inoltre l'attività di templato della cromatina verso le tre forme multiple di RNA polimerasi omologhe; tale capacità trascrittiva permette un'analisi della frequenza relativa dei siti di inizio della trascrizione sulla cromatina rispetto al DNA.

Abbiamo infine analizzato l'effetto delle proteine estratte dalla cromatina di lievito sulla trascrizione del DNA da parte delle tre forme separate di RNA polimerasi. I risultati mostrano che sia l'effetto di stimolazione che l'effetto di restrizione della trascrizione è funzione della quantità di proteine cromosomali presenti e che le tre polimerasi differiscono tra loro per le capacità di trascrivere strutture genetiche parzialmente ricostruite.

The study of gene expression in eukaryotes requires a satisfactory understanding of the regulatory role of chromatin. Our approach to this problem is to define some transcriptional characteristics of the chromatin of *Saccharomyces cerevisiae* used with homologous DNA–dependent RNA polymerases.

The study of yeast as a best-fit eukaryotic organism for trascriptional studies is now in progress in several laboratories, the rationale for such a choice relying on considerations of genetic and physiological manoeuvrability and on the presence of a relatively simple but complete set of eukaryotic attributes; in particular although the amount of DNA in yeast is far lower than the one of most animal cells, the presence of repetitive DNA has been reported in various species of yeast [1]; this is also true for *S. cerevisiae*, which has around 20 % of intermediate repetitive sequences (our unpublished results).

Transcription of chromatin has so far usually been studied making use of heterospecific systems, the template usually coming from animal sources and the RNA polymerase from *Escherichia coli*. The high-yield method worked out by Adman *et al.* [2] for the purification of yeast RNA polymerases and the presence in yeast of a chromatin-like material [3, 4] make possible the approach to the problem in a homologous system.

In this paper we present evidence that yeast chromatin contains endogenous RNA polymerase activity and that can act as template for the puri-

(*) Lavoro eseguito nel Centro di Studio per gli acidi Nucleici del C.N.R. e Cattedra di Biologia Molecolare, Istituto di Fisiologia Generale, Università di Roma.

(**) Pervenuta all'Accademia il 9 ottobre 1974.

fied RNA polymerases I, II and III. The activity ratio (synthesis on yeast chromatin *vs* synthesis on native calf thymus (CT) DNA) is different for each form of RNA polymerase; enzyme II in particular shows a quite low affinity for chromatin compared to the one shown for native CT DNA.

The use of the rifamycin derivative AF/013 allows us to calculate the amount of synthesis not due to " recycling " of the enzymes: we are thus able to estimate the relative frequency of initiation sites on chromatin and DNA.

We show furthermore that the proteins removed from yeast chromatin (previously termed π factor [3] and known to stimulate RNA synthesis mostly for enzyme III) can also restrict transcription differently for each of the three different enzymes.

Materials and Methods

Multiple RNA polymerases were purified from aerobically grown *Saccharomyces cerevisiae* S 41 as described in [2] to the DEAE Sephadex step. The reaction mixture for RNA polymerase assay was 100 mM Tris–HCl (pH 7.9), 3.2 mM $MnCl_2$, 1 mM ATP, CTP and GTP, 20% glycerol and contained 10 μCi [H^3] UTP/0.1 ml (13 Ci/mM Amersham Radiochemical Centre). The DNA used as aspecific template was from calf-thymus (type V–Sigma Chemical Co.) and, unless otherwise indicated, was used at a concentration of 100 μg/ml. Reactions were carried on for 20 min at 30° C and stopped by cold TCA precipitation.

The yeast chromatin material with template and RNA polymerase activity was purified by Agarose chromatography as described in [3]; the procedure for preparation of the chromatin proteins purified to the 1 M $(NH_4)_2SO_4$ sucrose gradient step (π factor) is *ibidem* described.

RNA polymerase chromatin and π factor were stored at —20° C in 50 % glycerol for several weeks. The semi-synthetic derivative of rifamycin AF/013 (a gift from Lepetit SpA, Milano, Italy) was kept at 4° C in dimethyl sulfoxide (DMSO) at 10 mg/ml for no more than one week.

Results and Discussion

The profile of elution from an Agarose column of the yeast chromatin is reported in fig. 1. In the lower part of the figure is shown the RNA polymerizing activity. Two peaks are easily distinguished: the first is not dependent upon addition of DNA, although it may be somewhat stimulated by exogenous DNA. The second peak is constituted by " free " enzyme, completely dependent upon added template. Free enzyme is mostly RNA polymerase II, as indicated by its activity ratio on native/denatured CT–DNA and its sensitivity toward α–amanitin [2], but is contaminated by other forms of RNA polymerase. The ratio " bound enzyme/free enzyme " is variable; the sen-

sitivity toward α-amanitin can also vary between 80 and 100 %; this variability makes, for the moment, of little interest and reliability the determination of the composition of the enzyme population present within the chromatin peak.

What we call " yeast chromatin " is the first peak; for further studies we collect the left side of this peak to avoid contamination with free enzyme.

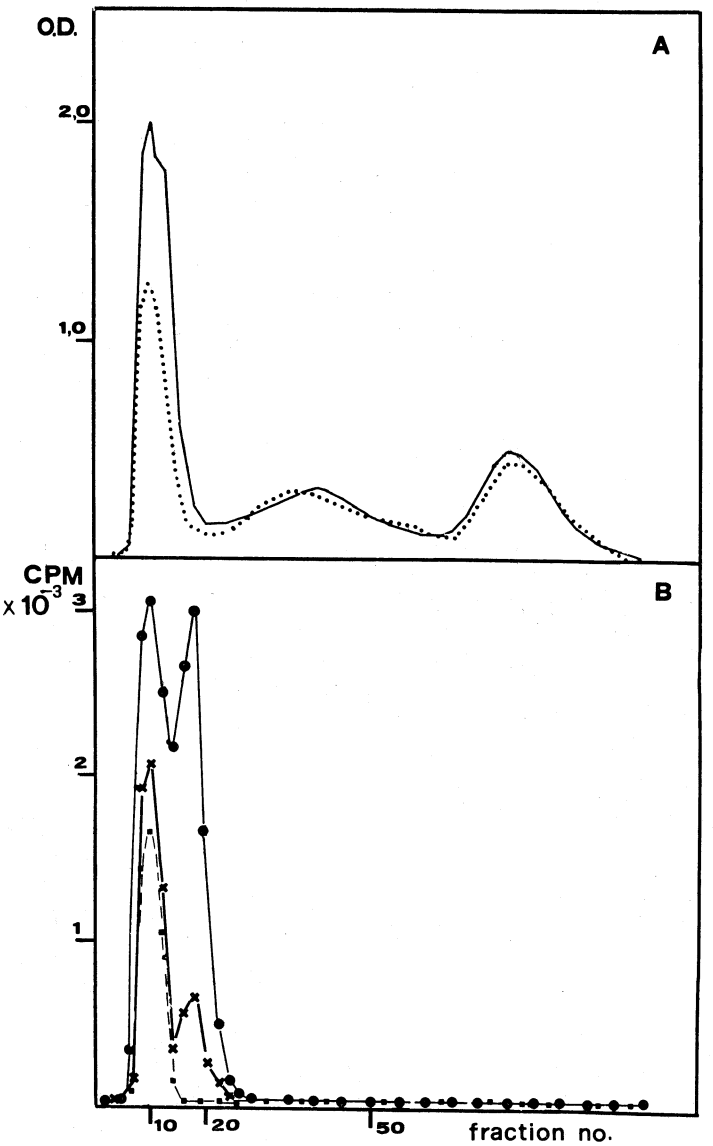

Fig. 1. – Agarose chromatography of yeast chromatin material. A) Optical density (———) at 260 mμ and (– – –) at 280 mμ. B) RNA polymerase activity assayed with standard methods. A final volume of 0.1 ml contained 50 μl of fraction and 50 μl of mix. Assays were run with 100 μg/ml denatured calf-thymus DNA (●——●), 100 μg/ml native calf-thymus DNA (×——×) or no added template (■——■).

From this material both low M.W. proteins and RNA polymerase can be easily extracted, as described in [3]; the influence of these proteins (termed π factor) on trascription is ibidem described. The behaviour toward the $(NH_4)_2SO_4$ concentration of this material has the pattern shown in fig. 2. Animal chromatin [5] shows a somewhat similar profile, with a second region of activity at high salt; the authors suggest that this second peak of activity might be due to the removal of proteins from chromatin by increasing ionic strength. An alternative explanation might be that RNA polymerase III,

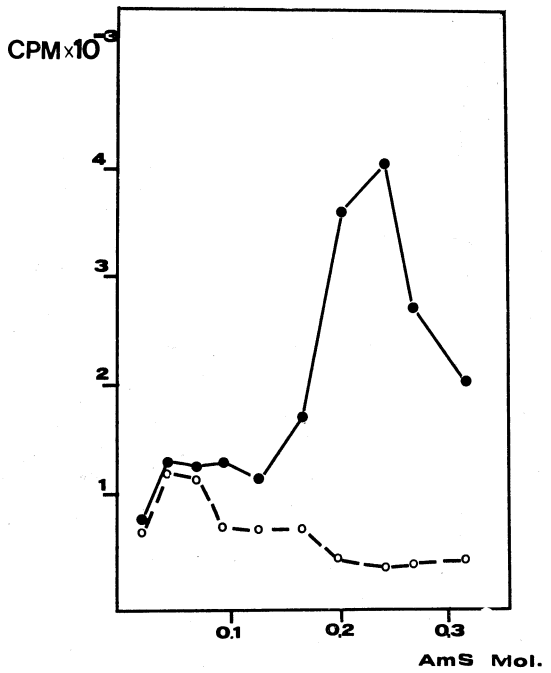

Fig. 2. – Endogenous RNA polymerase activity of yeast chromatin in function of the $(NH_4)_2SO_4$ concentration. Aliquots of 100 µl of chromatin were assayed with 50 µl/ml of AF/013 (o——o) or with no drug added (●——●) in a final volume of 0.2 ml with a standard reaction mixture containing the indicated concentration of $(NH_4)_2SO_4$.

the only active at high salt, is activated starting at 0.15 M $(NH_4)_2SO_4$ and reinitiates actively, becoming thus sensitive to AF/013. If indeed the incorporation assay is run in presence of 50 µg/ml of AF/013 (fig. 2) a region of resistance is observed at low salt, while at high salt the synthesis is almost completely inhibited. The sensitivity of the enzyme extracted from this material and tested on a heterologous template (experiment reported in fig. 3 curve C) is complete; the sensitivity of the endogenous activity of chromatin at low salt (fig. 3 curve B) is instead very low, indicating a low amount of reinitiation (sensitivity, from data obtained in similar experiments, varies between 5 and 25 %). It has been shown by Busiello *et al.* [6] that the rifamycin deriva-

tive AF/015 acts on the free molecules of RNA polymerase but not on the molecules that synthetize RNA: the drug, added at time 0, inhibits: if, instead, it is added when the synthesis has already begun, no inhibition is obtained. Meilhac *et al.* [7] have furthermore shown that the derivative used by us, AF/013, acts on the free molecules of RNA polymerase, on the molecules during the primary binding and on a further step of the initiation, but not on the elongation, of the chain.

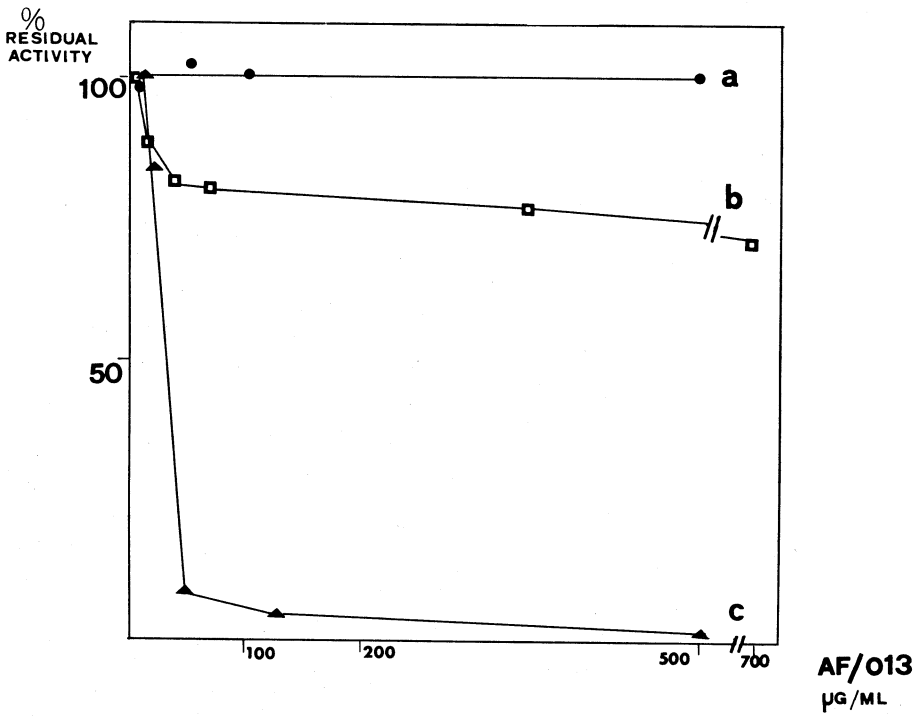

Fig. 3. – Sensitivity of endogenous or extracted RNA polymerase to AF/013. Curve *a* (●——●) shows the residual activity of RNA polymerase added to yeast chromatin; curve *b* (□——□) shows the sensitivity of the endogenous RNA polymerase of the yeast chromatin; curve *c* (▲——▲) shows the sensitivity of the RNA polymerase extracted from the chromatin as described in [3] and assayed on native calf-thymus DNA. The order of addition was: template, enzyme, 5 min, AF/013 (or DMSO as control), mixture.

It seems thus justified to use the drug AF/013 (as the use of rifamycin on prokaryotic RNA polymerase) to measure in a given system of synthesis the amount of RNA due to elongation of chain versus the amount due to the reinitiation of synthesis. The aspecific effect of protection by contaminating proteins [6, 7, 8] is in this case not relevant because even at a concentration of 25 mg/ml of AF/013 there is clear inhibition (fig. 3 curve 5) and, on the other hand, the inhibition, after reaching a maximum of 20 %, does not become much stronger with concentrations as high as 700 μg/ml. The resistance of the RNA polymerase activity of the chromatin toward α-amanitin (un-

shown data) is complete; we believe that this implies the absence of enzyme II from the chromatin.

The chromatin can work as a template for added enzyme. Fig. 3 curve a shows an experiment performed to test the sensitivity of the activity added to the chromatin; in this case the order of addition is template/enzyme/5 min/AF/013/Mix–XTP: one would thus [7] expect a sensitivity complete or almost complete of the enzyme. This is actually true if the template is calf–thymus native DNA (not shown); if the template is instead yeast chromatin the synthesis is completely resistant. As for the endogenous activity (fig. 3 curve *b*) also the added activity is resistant up to 500 μg/ml; these findings show that the resistance conferred to the enzyme is a characteristic of the chromatin or of one of his components. This noteworthy characteristic of conferring resistance toward a rif-derivative that acts on the initiation but not on the elongation has been previously reported for π factor which, being a mixture of protein components of yeast chromatin makes this finding somewhat expected. This indicates that initiation on chromatin is different from initiation on DNA at least for the step (or steps) sensitive to the drug.

The template activity of yeast chromatin was tested with three multiple RNA polymerases purified from yeast; in fig. 4 the results of one of such experiments are reported. RNA synthesis obtained was plotted against the increasing amounts of template (homologous chromatin or heterologous DNA) used; the results show that each enzyme reaches a different plateau for each different template. The difference in plateau value might be due to a different ability of each enzyme to reinitiate depending on the template; to ascertain this point we made use of the antibiotic AF/013 that, as mentioned above, blocks free enzyme, thus preventing reinitiation. For this control, the templates were incubated with the enzyme and a reaction-mixture lacking UTP to allow initiation but prevent an extensive elongation; after 15 min AF/013 or DMSO for control was added; after additional 5 min [H^3] UTP was added and the reaction carried out for 20 min. Synthesis not inhibited, that is the one not due to reinitiation, is 29, 17 and 44 % of the total activity respectively for enzyme I, II and III on DNA template, and 98,80 and 80% for the three enzymes on the chromatin. The difference is striking and indicates a high recycling of the polymerizing enzymes on DNA but not on chromatin; normalization of the plateau value for residual activity renders the saturation values obtained for each enzyme on the two templates very similar one to the other.

The other difference put in evidence by this kind of experiment is the amount of template necessary for reaching the saturation: around 10 μg/ml, of pure CT–DNA (= 14 nmoles of base pairs) are enough to reach the saturation for the three enzymes. The amount of DNA in chromatin form necessary for saturation is instead 7–9 times higher (100–130 nmoles of base pairs). The initiation sites on chromatin are thus 7–9 times more rare on chromatin than on DNA. This kind of calculation is of course valid only if the average length of the RNA chain synthesized on the two templates is equal; if, in

other words, the average length of the chains synthesized on chromatin were longer, one could infer that initiation sites on chromatin were still more rare. An analysis on sucrose gradients of the M. W. of the RNA synthesized by each enzyme on the two templates indicates an average length slightly higher

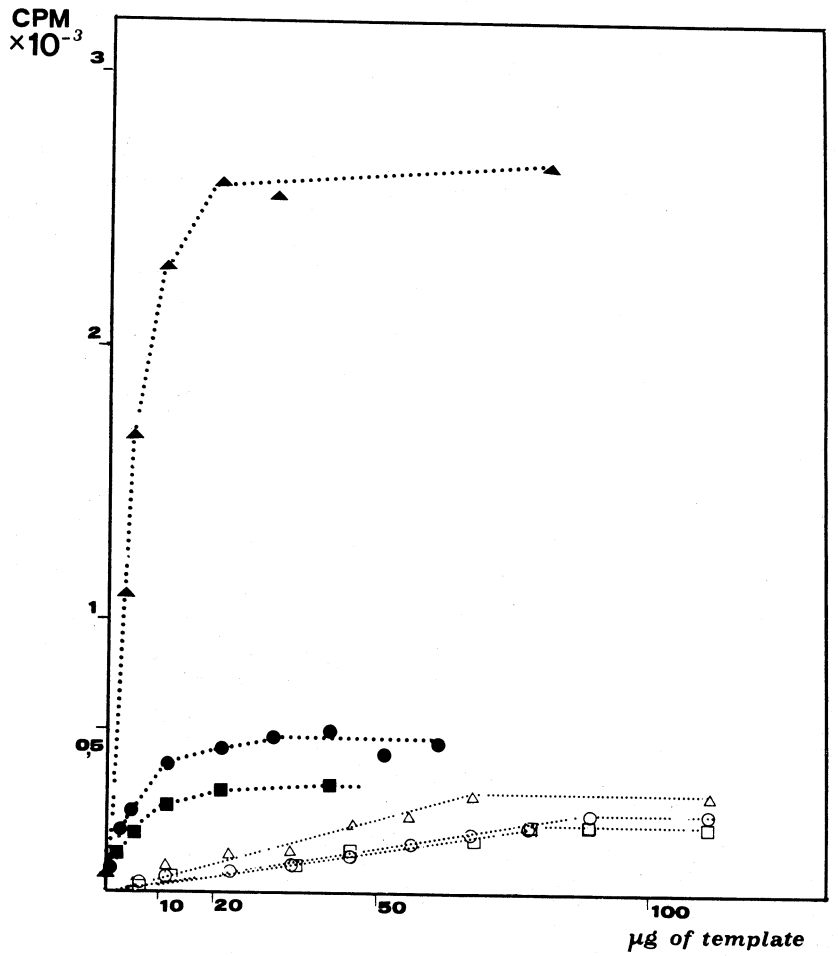

Fig. 4. – Template activity of native calf-thymus DNA and of yeast chromatin for the three separate RNA polymerases. 0.3 ml assays contained: 0.1 ml of the indicated enzyme, kept in 50% glycerol. 0.1 ml of standard mix lacking DNA and 0.1 ml of template. The final molarity of $(NH_4)_2SO_4$ in the assay was 25 mM for enzyme I, 35 mM for enzyme II, 50 mM for enzyme III.

(●——●) EI on DNA (o——o) EI on chromatin;
(▲——▲) EII on DNA (△——△) EII on chromatin;
(■——■) EIII on DNA (□——□) EIII on chromatin.

from chromatin templated RNA. This kind of measurements may bear an inner imprecision, possibly in the direction of overestimation of the chains synthesized on chromatin because there could be a background of RNA chains already initiated *in vivo* and only elongated by endogenous activity. The

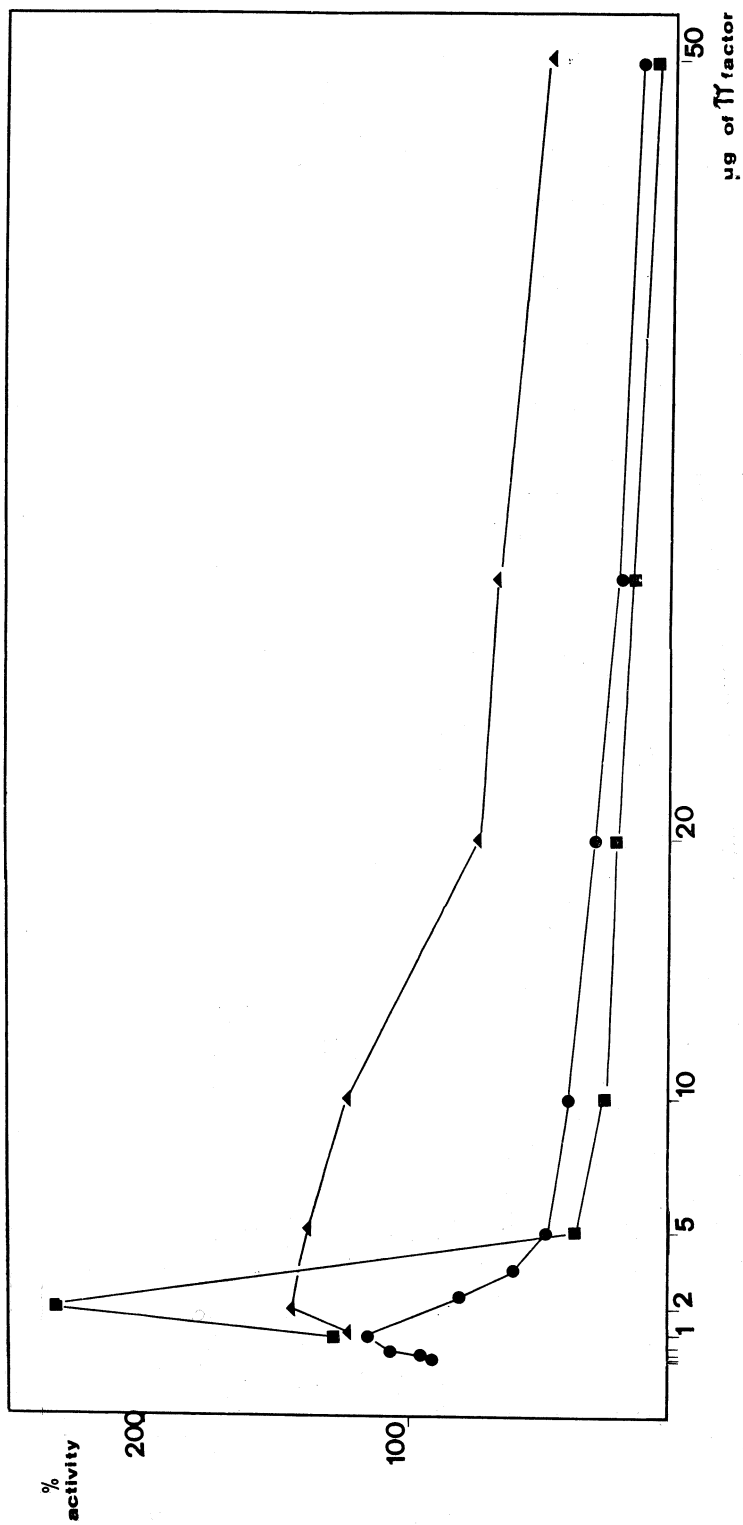

Fig. 5. – Transcription by three RNA polymerases on calf thymus native DNA in function of the amount of π factor. Each assay contained in a final volume of 0.3 ml and amount of Enzyme I (●——●), II (▲——▲), or III (■——■) that was saturated by the 30 μg of the native calf-thymus DNA present and the indicated amount of π factor. Final $(NH_4)_2SO_4$ concentration was 105 mM for EI, 115 for EII and 125 for EIII. The amount of DNA was fixed throughout the whole experiment. The order of addition was DNA π, enzyme, mixture.

low level of endogenous activity (not higher than a few % of the total) renders however this *imprecision* not very relevant). It seems thus justified to conclude that chromatin (as clearly shown by Felsenfeld in his *E. coli* RNA polymerase-CT chromatin system [9]) is endowed with ability to restrict transcription by offering to the enzyme a far lower number of initiation sites.

The question arises whether, as a first approach, it is possible to reconstruct *in vitro* with π factor [3] this ability to restrict, in addition to the one of stimulating already reported. In the experiment reported in fig. 5 we tested the effect of increasing amounts of π factor on the RNA synthesis carried out by fixed amounts of enzyme and fixed amounts of native CT-DNA. The results indicate a clear restriction effect; they also show that both the amounts of π necessary for optimal stimulation and the level of stimulation obtained in these conditions are different for each enzyme. The most trivial interpretation, that this effect is due to partial precipitation of DNA by histones present in π, is in our opinion unlikely because at a fixed ratio DNA/π (see for instance 5γ) one enzyme is stimulated while another is restricted and, on the other hand, it has been shown that solubility of the chromatin [9] is not an important factor for transcription.

At present not much may be said about the significance of this selective restriction effect, but in terms of what can be expected, we think that this is just what chromatin components should do: regulate—that is mostly prevent—the transcription of most of the message; in particular, if one attributes a different function to the different forms of RNA polymerase, one expects, as in this case, a different ability of each enzyme to transcribe a partially reconstructed structure.

Thanks are due to Lepetit SpA, Milano, for providing the rifamycin derivative AF/013.

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