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Transcription in Yeast: A Factor that Stimulates Yeast RNA Polymerases

(eukaryote/promoter recognition/rifamycin AF-013)

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ABSTRACT **Yeast cells contain an RNA polymerase factor, π, which is a heat-stable protein with an apparent molecular weight of 12,000. This factor stimulates transcription of calf-thymus, salmon-sperm, yeast-nuclear, and T4-phage DNA. It stimulates transcription by each of the four yeast-nuclear RNA polymerases, by rat-liver RNA polymerases I and II, and by *Escherichia coli* RNA polymerase. π-Factor can cause each of the eukaryotic RNA polymerases to become insensitive to rifamycin AF-013, but does not stop inhibition of *E. coli* RNA polymerase by rifamycin AF-013. Stimulation of transcription by π-factor is general, and does not apply only to a limited class of genes. Apparently, π-factor stimulates transcription by increasing the proportion of RNA polymerase binding events that leads to the initiation of RNA chains.**

Biochemical studies of transcription specificity in eukaryotic cells have been mainly focused upon two elements of the nuclear transcription apparatus: the DNA template and the enzyme that copies RNA from this template. Variations in the state of either element might affect gene activity. Experiments on chromatin preparations have emphasized the directing influence of chromosome structure upon gene expression. It has been proposed that various protein (1, 2) and RNA (3) molecules bound to chromosomal DNA specify active and inactive segments of the genome. The structural complexity of bacterial RNA polymerase (4) and the existence of multiple forms of eukaryotic RNA polymerase (5) suggest that gene activity may be regulated by variations in RNA polymerase. In developing prokaryotic cells a change in the structure of RNA polymerase can change the relative rate of transcription of different genes (6, 7). That similar events may occur during development of eukaryotic cells is suggested by the alterations in RNA polymerase profile observed during oogenesis in *Xenopus laevis* (R. Roeder, unpublished data) and *Strongylocentrotus purpuratus* (8).

The general applicability of template inactivation or sequestration theories (9) of gene control is challenged by the widespread occurrence of positive control in both eukaryotic (10) and prokaryotic (11) cells. The exposure of a bacteriophage gene to RNA polymerase frequently does *not* lead to transcription of that gene (12). Similarly, the absence of histone association is likely to be necessary, but not a sufficient condition, for the transcription of a eukaryotic gene. These arguments lead us to consider the role of a third class of transcription elements, including RNA polymerase factors and chromosomal proteins, that may increase the probability that RNA polymerase will transcribe a given region of DNA.

In eukaryotic cells, the identification of transcription factors is complicated by the presence of multiple forms of RNA polymerase within the cell nucleus. Multiple DNA-directed RNA polymerases of nuclear origin have been isolated from *Saccharomyces cerevisiae* (13–16). The 3 major enzymes observed correspond closely in their properties (16) to RNA polymerases I, II, and III isolated from animal-cell nuclei. Like mammalian polymerases I and II, these yeast polymerases are inhibited by rifamycin AF-013 (16, 17).

In the present communication, we describe a yeast protein that stimulates eukaryotic RNA polymerases. This factor resembles *Escherichia coli* sigma factor in having no permanent stimulatory effect upon the DNA template. Unlike any previously described RNA polymerase factor, the yeast factor protects eukaryotic RNA polymerases against the action of an RNA polymerase inhibitor: rifamycin AF-013. The factor stimulates *E. coli* RNA polymerase, but does not protect it against inhibition by rifamycin AF-013.

METHODS

Cell Growth, Disruption, and Fractionation of the Homogenate. S. cerevisiae S41 was grown aerobically to a density of 5-10 g/liter in a medium containing per liter 20 g of glucose, 20 g of peptone, and 10 g of yeast extract. To 20 g of washed cells, 20 g of glass beads (0.45 mm diameter) and 20 ml of homogenization buffer containing 50 mM Tris·HCl (pH 7.9)‡–10% glycerol–0.75 M $(NH_4)_2SO_4$–1 mM ethylenediaminetetraacetate (EDTA)–2 mM 2-mercaptoethanol, was added. The mixture was shaken for six periods of 30 sec in a Braun homogenizer with liquid CO_2 cooling. The homogenate was decanted, sonicated for four periods of 30 sec, and centrifuged at 36,000 rpm for 90 min in a Spinco type 60 Ti rotor. The supernatant was applied to a Bio-Gel Agarose A-1.5 m (100–200 mesh) column (100 × 3.2 cm) and eluted at 4° with buffer A, which is: 10 mM Tris·HCl–5% glycerol–0.05 M $(NH_4)_2SO_4$–1 mM EDTA–2 mM 2-mercaptoethanol (final pH = 7.9). Fractions of 6 ml were collected.

Assay for RNA Polymerase Activity and Stimulation by π-Factor. Standard incubation mixture contained: 50 mM Tris·HCl (pH 7.9), 1.6 mM $MnCl_2$, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 5 μCi [^{3}H]UTP (20 Ci/mmol) per ml, 100 μg of native calf-thymus DNA per ml, and enzyme. Reactions (0.1–0.5 ml) were started by warming the mixture to 30° and stopped after 30 min by cooling and trichloroacetic acid precipitation. Acid-insoluble counts were deter-

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‡ The pH values of all buffers in this paper were adjusted at room temperature (22–24°).

mined as described (16). π-Factor was added to the enzyme before the other constituents of the reaction mixture. Final salt concentration for factor assay was 0.1 M $(NH_4)_2SO_4$.

Sucrose-Gradient Centrifugation. The three top fractions of the first peak of RNA polymerase activity eluted from the agarose column (Fig. 1) were pooled and layered on six gradients containing 5–20% sucrose (w/v), 10% glycerol, 10 mM Tris·HCl (pH 7.9), 1 M $(NH_4)_2SO_4$, 1 mM EDTA, 2 mM 2-mercaptoethanol. Gradients (56 ml each) were centrifuged for 40 hr in a Spinco SW 25.2 rotor at 25,000 rpm at 4°. 2.3-ml Fractions were collected by siphoning. Factor activity was detected by measurement of the polymerizing activity of 20 μl of the RNA polymerase peak fraction in the presence of 20 μl of the fraction to be assayed in total volume 0.2 ml.

Ammonium Sulfate Precipitation. Fractions of the sucrose–1 M $(NH_4)_2SO_4$ gradients containing the RNA polymerase-stimulating activity were pooled, and $(NH_4)_2SO_4$ was added with slow stirring at 4° up to 65% saturation. The precipitate was collected by centrifugation for 10 min at 15,000 × *g* and solubilized in 1 ml buffer A, containing 1 M $(NH_4)_2SO_4$. This fraction is concentrated π-factor.

Yeast RNA Polymerases IA, IB, II, and III Fractionated on DEAE-Sephadex were isolated as described (16).

RESULTS

Isolation of Aggregate RNA Polymerase. A crude extract of yeast cells was prepared and loaded on an agarose column as described in *Methods*. Two partially resolved peaks of RNA polymerase activity were found in the column effluent fractions. The first (aggregate RNA polymerase) was completely excluded, whereas the second penetrated the gel slightly. Whereas soluble polymerase is dependent upon added DNA template (Fig. 1), aggregate polymerase contains a substantial DNA-independent activity, indicating the presence of template DNA in the excluded fractions. Material in those fractions containing yeast aggregate RNA polymerase complex resembles chromatin in its tendency to precipitate in medium with low salt concentration.

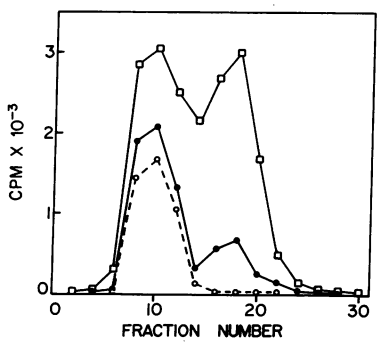

FIG. 1. Agarose chromatography of RNA polymerase activity present in a crude extract. Extraction and fractionation were as described in *Methods*. Aliquots of 50 μl were assayed in a final volume of 0.1 ml in the standard reaction mixture containing as DNA template: 10 μg of denatured calf-thymus DNA (□——□), 10 μg of native calf-thymus DNA (●——●), or no DNA template (○——○).

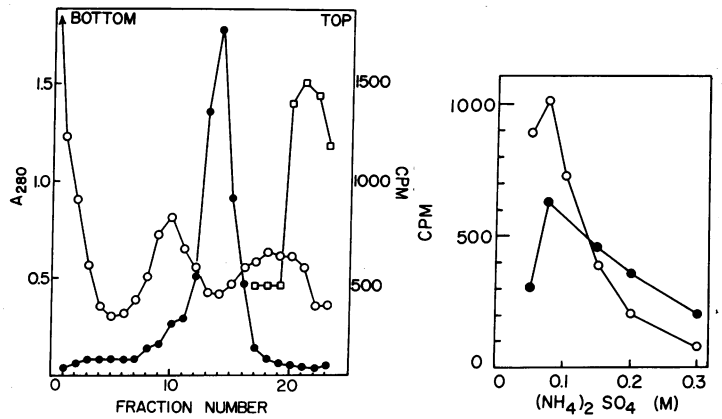

FIG. 2 (*left*). Release of factor activity from Agarose fractions sedimented in a 5–20% sucrose gradient containing 1 M $(NH_4)_2SO_4$. Procedure was as described in *Methods*. ○——○, absorption at 280 nm; ●——●, RNA polymerase assay; □——□, factor assay (uncorrected for activity of enzyme alone.)

FIG. 3 (*right*). Salt sensitivity of factor-stimulated RNA synthesis. RNA polymerase peak fractions from the sucrose–1 M $(NH_4)_2SO_4$ gradients (Fig. 2) were dialyzed against buffer A. 10 μl of dialyzed enzyme was incubated under standard conditions with 120 μg of native salmon-sperm DNA in a final volume of 1 ml containing $(NH_4)_2SO_4$ concentrations as indicated in the absence of factor (●——●) or in the presence of 50 μl from the pooled top fractions of the sucrose–1 M $(NH_4)_2SO_4$ gradients (○——○).

Release of a Stimulatory Factor. Exposure of the aggregate polymerase fractions to 1 M $(NH_4)_2SO_4$ released RNA polymerase in a soluble form (Fig. 2). The activity of this soluble RNA polymerase [1 M $(NH_4)_2SO_4$–RNA polymerase] is completely dependent upon added DNA template. To assay for the release of a stimulatory factor from aggregate polymerase, fractions from the 1 M $(NH_4)_2SO_4$ sucrose gradient were incubated with a constant amount of the 1 M $(NH_4)_2SO_4$–RNA polymerase peak fraction (Fig. 2). Fractions near the meniscus were found to markedly stimulate the 1 M $(NH_4)_2SO_4$–RNA polymerase fraction. We shall refer to this stimulating activity as the π-factor.

Physical and Chemical Properties of the π-Factor. (*i*) Both unstimulated activity and activity stimulated by π-factor of the 1 M $(NH_4)_2SO_4$–RNA polymerase fraction are maximal at a concentration of 0.075 M $(NH_4)_2SO_4$ (Fig. 3).

(*ii*) To determine whether π-factor activity resides in a molecule that is protein (partially or totally), we examined the effect of trypsin on factor activity. Proteolysis and assay for factor activity were performed separately and sequentially, with pancreatic trypsin inhibitor present during the RNA polymerase factor assay. Trypsin treatment completely destroyed π-factor activity; trypsin inhibitor had no influence on the RNA polymerase or the factor-stimulated reactions.

(*iii*) π-factor retains 60% of its stimulating activity after heating for 5 min at 80° in Buffer A, with 0.2 M $(NH_4)_2SO_4$.

(*iv*) Sucrose-gradient velocity sedimentation showed that the factor has about the same sedimentation rate as cytochrome *c* [molecular weight 12,400 (18)].

TABLE 1. *DNA template activity after treatment with π-factor*

Template	[³H]UMP incorporation (cpm) Without π-factor	With π-factor	Stimulation
Reisolated, pretreated with factor	2970	9035	3.0
Reisolated DNA	2760	9860	3.6
Untreated DNA	2710	8060	3.0

Native calf-thymus DNA was incubated for 30 min at 30° in polymerase assay mixture as described in *Methods*, except for the absence of [³H]UTP and enzyme, and the presence of an amount of concentrated π-factor that would give a 3-fold stimulation in a polymerase reaction. The DNA was purified by phenol extraction, dialyzed for 48 hr against 10 mM Tris·HCl (pH 7.5), and used as template for Enzyme III in a regular assay mixture with or without π-factor. As controls, template activity was determined for untreated calf-thymus DNA and for DNA purified as above, but previously incubated without the factor.

Time-Course of Stimulation. Comparison of rate of RNA synthesis with polymerase III and polymerase III with π-factor showed that π-factor stimulates both the initial rate and the duration of RNA synthesis.

π-Factor is Not a Nuclease. One of the ways in which a protein "factor" can stimulate RNA polymerase activity is by endonucleolytic cleavage of a double-stranded DNA template (19). Such single-strand interruptions facilitate initiation by the RNA polymerase of *E. coli* in the absence of sigma factor (19). If the yeast factor were acting to stimulate transcription in this way, we would expect to see an "activation" of DNA template by prior treatment with π-factor. DNA previously treated with π-factor had virtually the same template activity as the control DNA (Table 1), both in the presence and absence of π-factor.

Stimulation of Various Yeast RNA Polymerases by π-Factor. Our initial experiments with π-factor and 1 M $(NH_4)_2SO_4$–RNA polymerase fraction do not define either the function or localization of the factor in relation to the individual yeast RNA polymerases (IA, IB, II, and III). To test for function, we measured the ability of a limiting amount of π-factor to stimulate each of the polymerases. The reaction conditions were limiting enzyme on excess DNA. The results (Table 2) show that π-factor gives a large stimulation of the activity of RNA polymerases IB and III on native DNA, less stimulation of IA and II on native DNA, and little stimulation of the enzymes with a denatured DNA template.

The ability of π-factor to stimulate the yeast RNA polymerases eluted from DEAE-Sephadex (Table 2) suggests that these enzymes do not contain π-factor. Further evidence in support of this view was obtained by sedimenting polymerases IB, II, and III through sucrose gradients containing 1 M $(NH_4)_2SO_4$. This treatment did not appreciably change the degree of stimulation of any of the three enzymes by π-factor. Top fractions from these sucrose gradients contained no stimulating activity for 1 M $(NH_4)_2SO_4$–RNA polymerase. These experiments effectively demonstrate the absence of π-factor from DEAE-purified yeast RNA polymerases.

π-Factor Stimulation of Various DNA Templates. Because, in our standard assay for π-factor, similar results were obtained with calf-thymus, salmon-sperm, or yeast DNA as template, it is clear that the factor stimulation applies to eukaryotic DNA sequences that are not found exclusively in yeast DNA. As a further test of the generalized nature of π-factor stimulation, phage T4 DNA and poly(dA-dT) were tested as templates representing prokaryotic and simple synthetic DNA sequences. The results (Table 3) show that

TABLE 2. *Stimulation of separated yeast RNA polymerases by π-factor*

RNA polymerase	Calf-thymus DNA template	[³H]UMP incorporation (cpm) Without π	With π	Activity + π / Activity − π
IA	Native	700	1645	2.4
	Denatured	285	105	0.4
IB	Native	1430	4280	3.0
	Denatured	890	1360	1.5
II	Native	872	2375	2.7
	Denatured	1591	2415	1.5
III	Native	833	3970	4.8
	Denatured	652	815	1.3
1 M $(NH_4)_2SO_4$–enzyme	Native	570	2040	3.6

RNA synthesis and π-factor stimulation were assayed according to standard procedures in the presence of the following concentrations of $(NH_4)_2SO_4$: Enzyme IA, 83 mM; Enzyme IB, 102 mM; Enzyme II, 102 mM; Enzyme III, 106 mM, and 1 M $(NH_4)_2SO_4$–RNA polymerase, 100 mM. The π-factor used in these assays was one step further purified by carboxymethylcellulose chromatography (C. P. Hollenberg, unpublished data) and again concentrated by $(NH_4)_2SO_4$ precipitation. Assays with π-factor contained 5 μl of the concentrated preparation.

TABLE 3. *Stimulation of yeast RNA polymerase III by π-factor on different DNA templates*

Template	Relative quantity of factor	[³H]UMP incorporated (cpm)
No template	0	75
Calf-thymus DNA	0	2260
	2	3548
	5	3335
	10	3673
T4 DNA	0	448
	2	542
	5	717
	10	842
Poly(dA-dT)	0	4963
	2	6630
	5	6341
	10	5850

Reaction conditions: 100 μg of template DNA/ml, 0.1 M $(NH_4)_2SO_4$; reaction was for 30 min at 30° under standard assay conditions, with concentrated π-factor.

π-factor gives an appreciable relative stimulation of polymerase III activity on phage T4 DNA. On poly(dA-dT), which, unlike phage T4 DNA, is a rather active template for yeast RNA polymerase III, π-factor gives only a small stimulation.

Reversal of Rifamycin AF-013 Inhibition by π-Factor. Rifamycin AF-013 inhibits calf-thymus nuclear RNA polymerases I and II (17) and yeast RNA polymerases IB, II, and III (16). This drug may block initiation by eukaryotic RNA polymerases in the same way that rifamycin blocks initiation by bacterial polymerases. In each case, a limited number of polymerase molecules can bind to DNA to form preinitiation complexes resistant to rifamycin (20, 21), provided that the enzyme and DNA are incubated together before rifamycin is added. Because the yeast polymerases and the corresponding mammalian nuclear polymerases are similar both in their catalytic properties and rifamycin AF-013 inhibition curves (16, 20), we assume that this drug also inhibits yeast RNA polymerases before initiation, by binding to free enzyme molecules and preventing formation of a productive DNA–enzyme complex.

Surprisingly, addition of rifamycin AF-013 to a mixture of yeast RNA polymerase (IB, II, or III) and π-factor failed to produce inhibition of subsequent [^{3}H]UMP incorporation into RNA (Table 4). π-factor not only protected each of these RNA polymerases from inhibition by the drug, but also gave about the same stimulation as that produced by the factor in the absence of rifamycin AF-013.

Stimulation of Heterologous RNA Polymerases by Yeast π-Factor. The effect of π-factor upon transcription of calf-thymus DNA by *E. coli* RNA polymerase was measured both in the presence and absence of rifamycin AF-013. Table 5 shows that π-factor can stimulate *E. coli* polymerase more than six-fold. However, π-factor gives *E. coli* RNA polymerase no protection from inhibition by 30 μg of rifamycin AF-013 per ml and only slight protection from 1 μg of the drug per ml. Thus, in one of its activities, stimulation of transcription, π-factor fails to exhibit species-specificity while in another, protection against rifamycin AF-013 inhibition, the factor is able to distinguish between yeast and bacterial RNA polymerases.

TABLE 4. *Effect of rifamycin AF-013 on yeast RNA polymerase activity in the presence of π-factor*

	[^{3}H]UMP incorporation (cpm)			
	Without AF-013		With AF-013 (30 μg/ml)	
RNA polymerase	Without π	With π	Without π	With π
IB	2760	3700	785	3240
II	220	335	0	315
III	185	320	5	475
1 M $(NH_4)_2SO_4$–RNA polymerase	100	300	20	290

The reaction mixture contained, if indicated: 5 μl of polymerase enzyme, 5 μl of concentrated π-factor, 5 μl of rifamycin AF-013 (0.6 mg/ml, solubilized in ethanol), 0.1 M $(NH_4)_2SO_4$, and other reaction components in standard concentrations. Assays without rifamycin AF-013 contained 5 μl of ethanol. Final volume was 0.1 ml. The reaction constituents were added in the following sequence: enzyme, concentrated π-factor, rifamycin AF-013, standard reaction mixture.

TABLE 5. *Effect of rifamycin AF-013 on E. coli RNA polymerase activity in the presence of π-factor*

Enzyme	Concentrated π-factor (μl)	Rifamycin AF-013 μg/ml	pmol UMP incorp.	Stimulation or remaining activity
Yeast polymerase III	0	0	0.018	—
Yeast polymerase III	5	0	0.053	3.0
Yeast polymerase III	0	30	0.0009	0.05
Yeast polymerase III	5	30	0.034	2.0
E. coli polymerase (0.25 μg)	0	0	0.034	—
	5	0	0.137	4.0
	10	0	0.213	6.3
	0	30	0.002	0.05
	5	30	0.001	0.04
	10	30	0.005	0.16
E. coli polymerase (10 μg)*	0	0	1094	—
	0	0.5	227	0.21
	0	1.0	32	0.03
	0	2.5	9	0.01
	10	0	1491	1.36
	10	1.0	190	0.17
	10	2.5	74	0.06

Standard reaction conditions were used unless otherwise noted. The sequence of addition of components was as in Table 4. *E. coli* RNA polymerase was holoenzyme, containing the sigma factor. Yeast polymerase III was assayed as a control for the activity of π-factor and rifamycin AF-013.

* These reactions contained 10 mM $MgCl_2$ instead of $MnCl_2$ and 0.1 mM [^{3}H]UTP (50 Ci/mol).

In similar protection experiments with rifamycin AF-013, RNA polymerase I from rat liver was stimulated 2.8-fold by π-factor, either in the presence or absence of rifamycin AF-013; rat liver polymerase II was slightly stimulated by π-factor, but was completely protected from rifamycin AF-013 inhibition. In the absence of π-factor, both enzymes were 95% inhibited by the drug concentration used (50 μg/ml).

DISCUSSION

From the deoxyribonucleoprotein aggregate fraction of a crude yeast lysate we have isolated a small protein that stimulates transcription. Template-pretreatment experiments show that this protein must be present during transcription for the stimulation to occur, ruling out the possibility that π-factor is simply an enzyme that covalently modifies the DNA template.

The data we have obtained also seem to preclude another possible mode of factor action: the stimulation of transcription from a limited class of yeast genes. Because π-factor stimulates all four yeast nuclear polymerases, two mammalian polymerases, and *E. coli* RNA polymerase and, more importantly, because it stimulates transcription on T4 phage DNA as well as yeast, salmon-sperm, and calf-thymus DNA, it appears that this factor stimulates some aspect of transcription that is common to a large number of enzyme–template combinations.

The most striking property of π-factor is its ability to confer resistance to rifamycin AF-013 upon eukaryotic RNA polymerases that are normally inhibited by this drug. It appears that rifamycin AF-013 is an inhibitor of initiation by eukaryotic RNA polymerases. Therefore, we interpret the reversal of inhibition by rifamycin AF-013 by π-factor as meaning either that the factor binds to polymerase and blocks drug binding or that factor binding to DNA makes possible an alternative, rifamycin AF-013-insensitive mode of initiation.

In considering how the binding of π-factor to polymerase may stimulate transcription, it is useful to contrast the two differing ways in which RNA polymerase can bind to DNA. Productive binding events lead, most probably, to the initiation of RNA chains. For example, when *E. coli* holoenzyme binds to T4 DNA at 37° in 0.2 M KCl (22), productive binding results in initiation of chains at early phage T4 promoters. Largely unproductive binding of *E. coli* RNA polymerase has been observed under other conditions, notably at low-salt concentration (23). We hypothesize that π-factor stimulates transcription by shifting the balance between productive and unproductive binding events. The reasoning that leads to this hypothesis is the following: (*i*) The stimulatory effect of π-factor is seen only at low salt concentration [0.1 M $(NH_4)_2SO_4$ or less]. (*ii*) At low-salt concentrations, the number of *E. coli* RNA polymerase molecules bound to DNA (23) far exceeds the number (22) that can initiate RNA synthesis with high probability. (*iii*) Therefore, the conditions under which RNA polymerase binding to DNA is largely unproductive are the same conditions that allow maximum stimulation by π-factor. (*iv*) The activity of RNA polymerase measured with saturating amounts of a given DNA template apparently depends upon the proportion of RNA polymerase molecules that form productive complexes with that DNA. (*v*) One might therefore define a "good" template as one in which most of the binding events are productive. For yeast RNA polymerase III in 0.1 M $(NH_4)_2SO_4$, poly(dA-dT) is such a "good" template, giving a high activity at saturating concentrations of template. On this template, π-factor gives only a slight stimulation of transcription by yeast polymerase III.

Dependence of π-factor stimulation on salt concentration and template is consistent with the view that π-factor acts to increase the proportion of RNA polymerase-binding events that lead to initiation of transcription. In terms of detailed mechanism, π-factor might accomplish this by stabilizing complexes at promoter sites, by dissociating complexes on other DNA regions, or by creating new initiation sites at DNA sequences where bound RNA polymerase normally cannot initiate.

There is one further experimental finding that supports the view that π-factor brings about RNA polymerase binding to certain DNA sequences. In preliminary experiments, we have observed (C. P. Hollenberg, unpublished observation) that π-factor is readily bound to DNA under the ionic conditions used for *in vitro* transcription. Thus, there may be present, within an apparently rather small π protein molecule, binding sites both for RNA polymerase and for a particular DNA sequence or backbone conformation.

The one observation that would seem at first inconsistent with our hypothesized role for π-factor is its effect on *E. coli* RNA polymerase. Here π-factor is able to stimulate transcription, yet it does not protect the enzyme against inactivation by rifamycin AF-013. This inhibitory action of rifamycin AF-013 on *E. coli* polymerase despite the presence of π-factor is the main evidence that π-factor does not protect yeast polymerases by directly inactivating rifamycin AF-013. We explain the difference between π-factor action upon yeast and *E. coli* RNA polymerase by proposing that yeast RNA polymerases (as a group) and *E. coli* RNA polymerase differ in relative affinity for binding of π-factor *vis-a-vis* binding of rifamycin AF-013. Consequently, π-factor may bind to *E. coli* polymerase tightly enough to stimulate transcription, but not tightly enough to block rifamycin AF-013 binding to free polymerase.

If π-factor does indeed function generally in promoter recognition, both the existence of this protein and its ability to stimulate transcription on heterologous DNA will have a number of important practical and theoretical consequences.

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