On the Mode of Action of Lomofungin, an Inhibitor of RNA Synthesis in Yeast†

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ABSTRACT: Lomofungin is a potent inhibitor of RNA synthesis in yeast. Studies on the mode of action of the inhibitor were carried out using yeast RNA polymerases A and B and bacterial RNA polymerase. In vitro inhibition of RNA synthesis is independent of the nature and concentration of the template used and of the nucleoside triphosphate concentration. The extent of inhibition is strongly dependent upon the nature and concentration of divalent cations used to stimulate transcription. The three RNA polymerases were inhibited to the same extent in the presence of Mn^{2+} ions whereas little inhibition was observed with Mg^{2+} ions. Spectrophotometric studies reveal the formation of different complexes between lomofungin and divalent cations (Mn^{2+}, Mg^{2+}, or Zn^{2+}) with the respective stoichiometries of 0.5, 1, and 2 divalent cations per molecule of lomofungin. The complexes formed depend upon the nature of the divalent cation involved. No direct interaction between lomofungin and DNA could be observed in the presence of divalent cations but evidence is presented that lomofungin interacts with yeast RNA polymerase A. Inhibition of RNA synthesis occurs at the level of both chain initiation and elongation.

The role and relationship of the multiple forms of RNA polymerase discovered in eucaryotic organisms (Roeder and Rutter, 1969) could be clarified with the use of specific inhibitors acting at the level of the enzymes. The phenazine inhibitor lomogungin, unlike α-amanitin (Dezelee et al., 1970), enters the yeast cells and inhibits primarily RNA synthesis (Gottlieb and Nicolas, 1969; Kuo et al., 1973; Cannon et al., 1973). The drug has also been reported to be active in vitro on yeast and bacterial RNA polymerases (Cano, et al., 1973). Interestingly, Fraser et al. (1973) and Kuo et al. (1973) showed that lomofungin selectively inhibits the synthesis of rRNA and polydisperse RNA, whereas the synthesis of 5S and tRNA is practically unaffected, at least in the early period after drug addition (Cannon and Jimenez, 1974). We have investigated the mode of action of lomofungin on RNA synthesis using purified yeast RNA polymerases A and B as well as *Escherichia coli* RNA polymerase, in order to compare the in vitro and in vivo effects of the antibiotic. A strong correlation would provide a tool for inhibition of a specific RNA polymerase activity in the yeast cell. Also, if lomofungin were a specific inhibitor of yeast RNA polymerases, it might advance our understanding of the mechanism of action of these enzymes. It is shown that inhibition of RNA synthesis depends on the nature of the divalent cation used to stimulate transcription. Spectrophotometric studies show that lomofungin forms metal chelates with Mn^{2+}, Mg^{2+}, and Zn^{2+}. In addition, evidence is presented in favor of a direct interaction between lomofungin and RNA polymerase.

Materials and Methods

RNA Polymerases. DNA-dependent *Escherichia coli* RNA polymerase (Darlix et al., 1969), yeast RNA polymerase A (Buhler et al., 1974), and RNA polymerase B (Dezelee and Sentenac, 1973) were purified as previously described. The enzymes were homogenous proteins as judged by gel electrophoresis under nondenaturing conditions (Buhler et al., 1974).

Nucleic Acids and Nucleotides. Native calf-thymus DNA obtained from Sigma was purified on a nitrocellulose column (Dezelee and Sentenac, 1973). $d(A\text{-}T)_n$, $d(I\text{-}C)_n$, and $(rC)_n$ were obtained as previously described (Dezelee et al., 1974). [γ-^{32}P]GTP was prepared by the method of Glynn and Chappell (1964) and obtained from C.E.N. (Saclay) as were the other labeled nucleotides. Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals.

Lomofungin was generously supplied by Dr. Whitfield from Upjohn Co. The purity of the compound was checked by mass spectrometry. A stock solution was prepared by dissolving the dye in a minimal amount of 0.1 *N* NaOH (50 μl/250 μg of lomofungin) and stored at 4°C in a buffer containing 70 m*M* Tris-HCl (pH 8.0). The concentration of lomofungin was estimated spectrophotometrically using a molar absorption coefficient at pH 8.0 of 32,050 at 347 nm and of 31,800 at 270 nm in the above buffer.

Spectrophotometric Measurements. The interaction between lomofungin and different divalent cations was followed by direct or differential spectroscopy, using a Cary 14 or a Cary 15 recording spectrophotometer. The concentration of the different metallic salts ($MnCl_2$, $MgCl_2$, or $ZnSO_4$) was increased stepwise by addition of aliquots of the appropriate metal ion solution (5 μl) to a 3-ml solution of lomofungin in 70 m*M* Tris-HCl (pH 8.0). Final dilution of the lomofungin solution was less than 5%. The absorption spectrum of the solution was recorded after each addition of salt. Addition of an equivalent amount of EDTA, at the end of the entire titration procedure, rapidly and reversibly restored the spectrum of the original lomofungin solution. In no case was a precipitate of lomofungin observed in the absence or in the presence of divalent cations. Difference spectra were read against a reference cell containing the initial solution of lomofungin to which an equal volume of water was added to eliminate the dilution effect. This method allowed a better determination of the λ_{max} of the various complexes observed. The results are expressed as a function

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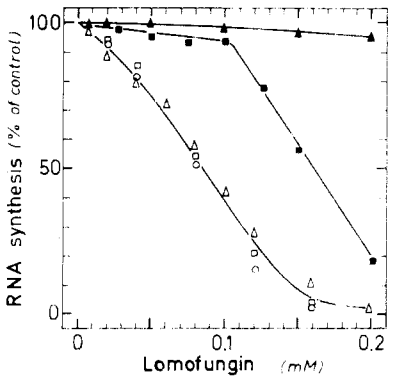

FIGURE 1: Effect of lomofungin on RNA synthesis by yeast and *E. coli* RNA polymerases. Yeast RNA polymerase A (1.7 μg), RNA polymerase B (1.8 μg), or *E. coli* holoenzyme (2 μg) was assayed using the standard assay mixture described under Materials and Methods with 12 μg of native calf-thymus DNA as template and in the presence of varying concentrations of lomofungin. RNA synthesis was measured after 30 min of incubation at 30°C. Results are expressed as percent of [^{3}H]UMP incorporation in the absence of lomofungin: RNA polymerase A (□), 0.23 nmol; RNA polymerase B (○), 0.23 nmol; *E. coli* enzyme (△), 0.95 nmol. Alternatively *E. coli* RNA polymerase and yeast RNA polymerase A were incubated under their usual assay conditions (see Materials and Methods) (i.e., with, respectively, 10 and 5 m*M* $MgCl_2$ instead of 3 m*M* $MnCl_2$ as in the above experiment). Under these conditions control incorporation of [^{3}H]UPM was 0.95 nmol with *E. coli* RNA polymerase (▲) and 0.4 nmol with RNA polymerase A (■).

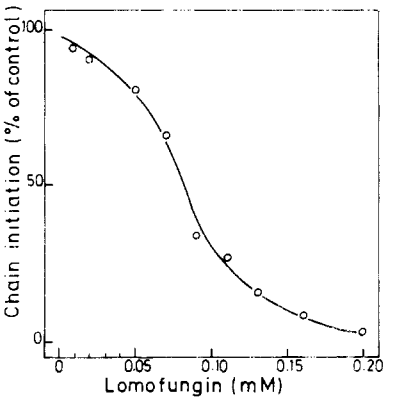

FIGURE 2: Effect of lomofungin on RNA chain initiation. Standard mixtures (0.2 ml), containing 1.3 μg of yeast RNA polymerase B and 15 μg of denatured DNA, were incubated with [γ-^{32}P]GTP as labeled nucleotide and varying concentrations of lomofungin. The inhibitor was added before the enzyme and reaction was started by the addition of Mn^{2+}. After 30-min incubation at 30°C, RNA chains were recovered by acid precipitation as described under Materials and Methods. Incorporation of [γ-^{32}P]GTP was 3.5 pmol in the absence of inhibitor.

of *r* which is the molar ratio of divalent cation to lomofungin. For spectrophotometric titration of lomofungin and p*K* determination, pH measurements were carried out with a Radiometer PHM 64 pH meter, before and after recording each spectra.

Polymerization Assay. A standard incubation mixture (0.2 ml) contained 70 m*M* Tris-HCl (pH 8.0), 5 m*M* dithiothreitol, 3 m*M* $MnCl_2$, 50 m*M* ammonium sulfate, 0.4 m*M* ATP, GTP, CTP, and[^{3}H]UTP (10,000 to 15,000 cpm/nmol), native or denatured calf-thymus DNA as indicated (10 μg), and yeast RNA polymerase B (1–2 μg). Unless otherwise indicated, lomofungin was added before the enzyme at 0°C and the reaction was started by the addition of $MnCl_2$. After 30 min at 30°C, the synthesized RNA was recovered by acid precipitation and its radioactivity was estimated as previously described (Dezelee and Sentenac, 1973). For chain initiation studies, 0.1 m*M* [γ-^{32}P]GTP was added as labeled nucleotide and the RNA chains were collected by acid precipitation (Dezelee and Sentenac, 1973). When indicated, yeast RNA polymerase A (Buhler et al., 1974) and *E. coli* RNA polymerase (Darlix et al., 1969) were alternatively assayed under their previously described assay conditions.

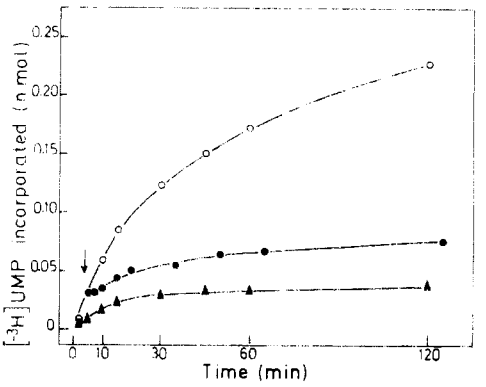

FIGURE 3: Inhibition of RNA synthesis by lomofungin added before or after the start of transcription. Three scaled up standard mixtures (1 ml) containing 12 μg of RNA polymerase B and 200 μg of native DNA were incubated with or without 0.11 m*M* lomofungin added either at zero time or after 5 min of synthesis. Aliquots (100 μl) were taken at different times and the RNA was recovered as usual: (○) control [^{3}H]UMP incorporation; (▲) lomofungin at 0 time; (●) lomofungin at 5 min.

Results

Inhibition of RNA Synthesis by Lomofungin. RNA synthesis was carried out in the presence of varying concentrations of lomofungin using yeast RNA polymerases A or B or *E. coli* enzyme, under their respective optimal conditions of transcription (Figure 1). The results clearly show a preferential inhibition of yeast RNA polymerase B and to a lesser extent of RNA polymerase A, whereas the bacterial enzyme was practically unaffected by up to 0.2 m*M* lomofungin. To see whether this differential inhibition was due to the different conditions of transcription used or reflected a specific inhibition of RNA polymerase B, the enzymes were assayed in the presence of lomofungin, under standardized conditions. Using the assay conditions of RNA polymerase B, which has an absolute requirement for Mn^{2+} ions (Dezelee and Sentenac, 1973), an identical inhibition of the three enzymes was obtained (Figure 1). The concentration of lomofungin required for 50% inhibition was about 0.08 m*M*, while complete inhibition was observed at about 0.2 m*M*. This result clearly indicated that the inhibitor had no particular specificity with respect to the various RNA polymerases used, and rather suggested that ionic conditions markedly influence the inhibitory action of lomofungin.

Effect of Lomofungin on RNA Chain Initiation and Elongation. When lomofungin was added at the start of transcription the inhibition could be accounted for by a decrease in the number of chains initiated (Figure 2). The incorporation of [γ-^{32}P]GTP was inhibited to the same extent as total RNA synthesis, at all concentrations of lomofungin used (compare Figures 1 and 2). The kinetics of RNA synthesis in the presence of lomofungin is shown in Figure 3. The inhibitor was added either at zero time, before RNA polymerase B, or after 5 min of synthesis, in order to investigate its effect on the chain elongation process. It is apparent that the extent of inhibition is independent of the time

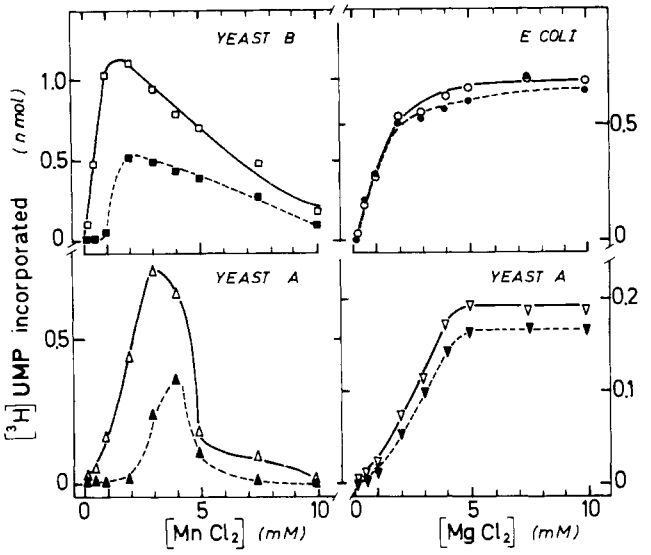

FIGURE 4: Effect of divalent cations on the inhibition of RNA synthesis by lomofungin. Yeast RNA polymerases A (2 μg) or B (1.5 μg) were assayed using the standard mixture described under Materials and Methods, with 10 μg of denatured calf-thymus DNA as template and varying concentrations of Mn^{2+} or Mg^{2+} as indicated. *E. coli* RNA polymerase (1.5 μg) was incubated under the same conditions but with 6 μg of native DNA. Incubation was carried out for 30 min at 30°C with or without 0.10 m*M* lomofungin: (open symbols) control synthesis in the absence of lomofungin; (filled symbols) RNA synthesis with 0.1 m*M* lomofungin.

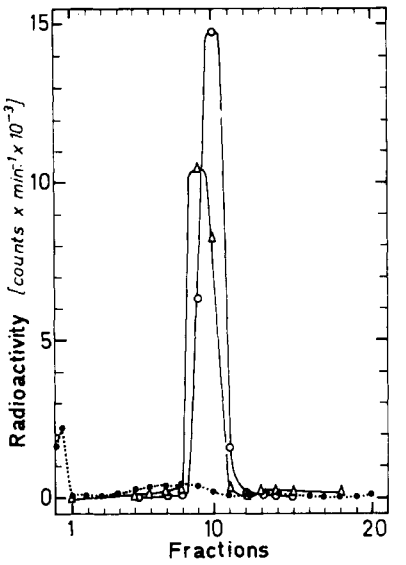

FIGURE 5: Interaction between RNA polymerase A and lomofungin in the presence of Mn^{2+}. RNA polymerase A (20 μg) and lomofungin (0.3 m*M*) were mixed in 0.2 ml of a buffer containing 3 m*M* $MnCl_2$, 70 m*M* Tris-HCl (pH 8), 5 m*M* dithiothreitol, and 50 m*M* ammonium sulfate. After 2 min at 30°C the mixture was layered on top of 5 ml of a glycerol gradient (10–30%) containing 20 m*M* Tris-HCl (pH 8.0), 10 m*M* 2-mercaptoethanol, 1 m*M* EDTA, and 50 m*M* ammonium sulfate. Centrifugation was carried out at 4°C, in an SW-65 rotor, for 4 hr at 65,000 rpm. Fractions of 0.25 ml were collected and assayed for RNA polymerase activity on 25-μl aliquots with denatured calf-thymus DNA as template: (○) RNA polymerase incubated without lomofungin; (●) RNA polymerase with lomofungin and $MnCl_2$; (△) RNA polymerase with lomofungin but without $MnCl_2$.

of drug addition, indicating that the drug inhibits the growth of initiated chains, as previously observed by Cano et al. (1973) using bacterial RNA polymerase. These results suggest that the inhibitor blocks the transcription complex probably by interacting with RNA polymerase in the process of chain initiation as well as elongation.

Influence of Divalent Cations. The effect of alterations in the Mn^{2+} concentration on the inhibition by lomofungin (0.10 m*M*) of yeast RNA polymerase A or B was investigated (Figure 4). In both cases, a complete inhibition of transcription was found at suboptimal concentrations of Mn^{2+}. Increasing Mn^{2+} ion concentrations above this threshold markedly reduced the inhibition. It should be noted, however, that addition of an excess of Mn^{2+} ions did not fully suppress the residual inhibition. On the other hand, with Mg^{2+} ions, practically no inhibition of RNA polymerase A was obtained in the presence of the same concentration of lomofungin (0.10 m*M*). Similarly, *E. coli* RNA polymerase was practically unaffected by 0.10 m*M* lomofungin at all the concentrations of Mg^{2+} ions used (from 0.5 to 10 m*M*). However, as indicated above, the bacterial enzyme proved as sensitive to lomofungin as the yeast enzymes in the presence of Mn^{2+} ions (see Figure 1). These results show that the nature and concentration of the divalent cation involved during transcription influence the inhibition brought about by lomofungin.

Influence of Template and Nucleotide Concentration. The fact that inhibition of transcription was observed with a 10- to 20-fold molar excess of Mn^{2+} over lomofungin suggested that the drug did not merely lower the concentration of free divalent cations in the incubation medium and rather suggested a possible interaction of the lomofungin–divalent cation complex with one component of the transcription system. Varying the template over a wide range of concentrations from 2 to 75 μg/ml did not affect the degree of inhibition. Identical dose–response curves were obtained with widely different templates such as native or denatured calf-thymus DNA, $d(A\text{-}T)_n$, or $(rC)_n$. These results, together with the spectral studies described below, suggested that lomofungin did not interact with the template. Similarly, no effect of nucleotide concentration was found on the extent of inhibition as previously observed by Cano et al. (1973). Using $d(I\text{-}C)_n$ as template, the apparent K_m for GTP and CTP remained unchanged (0.1 and 0.07 m*M*, respectively) in the presence of lomofungin (0.07 m*M*).

Interaction of Lomofungin with Yeast RNA Polymerases. Previous experiments of Cano et al. (1973) suggested an interaction of lomofungin with bacterial RNA polymerase. With yeast RNA polymerases A or B, increasing levels of RNA polymerase, from 5 to 50 μg/ml, barely reduced the inhibition. However, addition of high concentrations of serum albumin, up to 0.3 mg/ml, progressively lowered the inhibition, almost twofold, indicating a possible drug–protein interaction; on the other hand, other unrelated proteins, such as aspartate transcarbamylase (a zinc containing enzyme), lysozyme, or phosvitin, had no effect on lomofungin inhibition. The possible interaction of lomofungin and RNA polymerase was further investigated with or without divalent cations. A mixture of 0.3 m*M* lomofungin and RNA polymerase A was preincubated for 2 min at 30°C and then sedimented in a glycerol gradient to remove free inhibitor. The enzymatic activity recovered after centrifugation was compared to a control experiment without inhibitor (Figure 5). When lomofungin alone was preincubated with the enzyme, about 80% of the enzymatic activity sedimented like the control enzyme. On the other hand, when preincubation was carried out in the presence of Mn^{2+} ions no activity could be recovered at the level of the enzyme peak. A small percentage of the initial activity only was observed at the

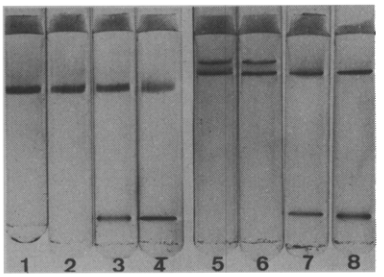

FIGURE 6: Effect of lomofungin on the electrophoretic migration of RNA polymerases A and B. Electrophoresis of RNA polymerases was carried out in 5% polyacrylamide gels under nondenaturing conditions (Dezelee and Sentenac, 1973). The enzymes (8 μg) were incubated for 2 min at 30°C with 0.3 mM lomofungin with or without 3 mM $MnCl_2$. After migration, the protein bands were stained with Coomassie Brilliant Blue. Free lomofungin and the lomofungin–Mn^{2+} chelate migrate with the front: (1) RNA polymerase B; (2) RNA polymerase B plus $MnCl_2$; (3) RNA polymerase B plus lomofungin; (4) RNA polymerase B plus $MnCl_2$ plus lomofungin; (5) RNA polymerase A; (6) RNA polymerase A plus $MnCl_2$; (7) RNA polymerase A plus lomofungin; (8) RNA polymerase A plus $MnCl_2$ plus lomofungin.

bottom of the centrifugation tube. The results therefore indicated that a lomofungin–Mn complex can interact with RNA polymerase A. In the above experiment, the glycerol gradient contained 1 mM EDTA which apparently did not interfere with the interaction during the course of the experiment. Interaction between lomofungin and RNA polymerase B was also suggested by glycerol gradient sedimentation. However, the effect was less drastic than with RNA polymerase A since about 50% of the enzymatic activity was recovered, sedimenting at the level of the control enzyme. Inhibition required the presence of $MnCl_2$ and was not observed when EDTA was added in the glycerol gradient.

To quantify the interaction between lomofungin and RNA polymerase A, a mixture of enzyme lomofungin and Mn^{2+} ions was chromatographed on Sephadex G 25. An enzyme–lomofungin–Mn^{2+} complex was obtained in the void volume, well separated from free dye. The presence of the lomofungin–Mn^{2+} complex, associated to the enzyme, was evidenced by its characteristic absorption spectrum in the visible range (see the spectral studies presented below). An average of 20 molecules of lomofungin was found per molecule of enzyme (assuming a mol wt of 500,000 for RNA polymerase A (Buhler et al., 1974)). The effect of lomofungin on RNA polymerases A and B was also investigated by gel electrophoresis (Figure 6). Lomofungin, with or without Mn^{2+} ions, did not interfere with the electrophoretic migration of RNA polymerase B. In contrast, the migration of RNA polymerase A was markedly altered by lomofungin. It was previously shown that this enzyme migrates in two enzymatically active bands of protein (Huet et al., 1975). The fast component, called RNA polymerase A*, differs from the normal enzyme by the absence of two subunits. After addition of lomofungin, either with or without Mn^{2+} ions, only the rapidly migrating fraction was seen in the gel (Figure 6). The result suggests that lomofungin favors the dissociation of RNA polymerase A. Interestingly, the same effect was observed with dithizone, a zinc chelating agent, but not with EDTA or 8-hydroxyquinoline.

Interaction of Lomofungin with Mn^{2+} and Other Divalent Cations. To proceed further, it was of interest to investigate and quantify the interaction of lomofungin with divalent cations. Divalent cations markedly altered the spectral properties of lomofungin at pH 8.0. Figure 7 is a comparison of the absorption spectra in the visible range which were obtained when Mg^{2+}, Mn^{2+}, or Zn^{2+} ions were added by increments to a constant concentration of lomofungin. A continuous spectral shift was obtained with increasing concentrations of divalent cations, showing three series of isosbestic points, clearly corresponding to three successive equilibria. One series of isosbestic points was found, for instance, with Mg^{2+} ions at 368, 390, 459, and 524 nm, at a molar ratio of Mg^{2+} to lomofungin ranging from $r = 0$ to $r = 0.5$ (Figure 7). A second series of isosbestic points was found between $r = 0.5$ and $r = 1$. The third one was found between $r = 1$ and $r = 2$. Similar results were obtained with

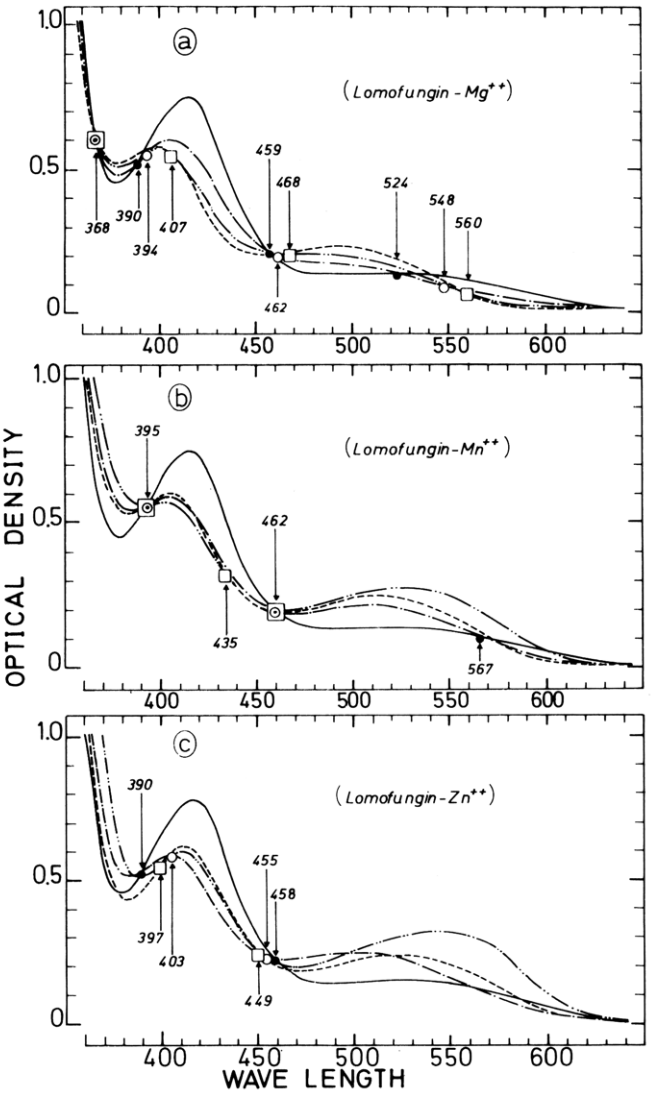

FIGURE 7: Complex formation between lomofungin and Mg^{2+}, Mn^{2+}, or Zn^{2+}. (a) The spectrum of 58 μM lomofungin in 70 mM Tris-HCl buffer was recorded after addition of Mg^{2+} as described under Materials and Methods. The spectra in the visible range are shown at the following molar ratio of Mg^{2+} vs. lomofungin: (—) $r = 0$; (- · -) $r = 0.52$; (- · · -) $r = 1.04$; (- - -) $r \geq 2.13$. (b) Absorption spectra obtained at the following molar ratios of Mn^{2+} vs. lomofungin: (—) $r = 0$; (- · -) $r = 0.45$; (- · · -) $r = 1.0$; (- - -) $r > 2$. Isosbestic points shown come from a family of such spectra obtained for $0 < r < 100$: (●) isosbestic points obtained between $r = 0$ and $r = 0.5$; (○) isosbestic points between $r = 0.5$ and $r = 1$; (□) isosbestic points between $r = 1$ and $r = 2$. (c) Absorption spectra obtained at the following molar ratio of Zn^{2+} vs. lomofungin: (—) $r = 0$; (- · -) $r = 0.46$; (- · · -) $r = 1.03$; (- - -) $r \geq 2.0$. Isosbestic points shown come from a family of spectra obtained in the range $0 < r < 100$: (●) isosbestic points obtained between $r = 0$ and $r = 0.5$; (○) isosbestic points obtained between $r = 0.5$ and $r = 1$; (□) isosbestic points between $r = 1$ and $r = 2$.

Mn^{2+} or Zn^{2+} instead of Mg^{2+} ions (Figure 7), although the spectral shifts were qualitatively different. In Figure 8 are shown the absorption curves as a function of r, at four different wavelengths (where the maximal spectral shifts were recorded), in the presence of Mg^{2+} ions. Three successive inflection points were observed at the four wavelengths mentioned, indicating the existence of three distinct complexes between Mg^{2+} ions and lomofungin, of stoichiometries of 0.5, 1, and 2 divalent cations per molecule of lomofungin. Similarly, three complexes were clearly observed with Mn^{2+} (or Zn^{2+}) with the same stoichiometry (Figure 8). In fact, several observations indicated that the nature of the complex formed between lomofungin and the three divalent cations, Mg^{2+}, Mn^{2+}, and Zn^{2+}, was different. First, the spectra of the three complexes observed at r = 0.5, 1, and 2 depended upon the nature of the divalent cation involved and were very different with Mg^{2+}, Mn^{2+}, and Zn^{2+} ions (see Figure 7). The spectral shifts observed with increasing concentrations of divalent cations were also different. For instance, the hypsochromic shift observed in the spectrum of lomofungin at r = 0.5 with Mg^{2+} ions was maximum around 480 nm and was more important than the spectral shift brought about by Mn^{2+} (maximum around 510 nm). When increasing the divalent cation concentration from r = 0.5 to r = 1 a strong bathochromic shift and hyperchromic effect were found with Mn^{2+} and Zn^{2+} ions (λ_{max}^{Mn} 540 nm; λ_{max}^{Zn} 550 nm; solution pink-purple). On the other hand, the corresponding spectral shifts were relatively much smaller in magnitude with Mg^{2+} (λ_{max}^{Mg} 490 nm; solution yellow). Going from r = 1 to r = 2, the spectrum of the lomofungin-Mn or -Zn complex presented a marked hypsochromic shift, with a slight hypochromic effect (λ_{max}^{Mn} 515 nm; λ_{max}^{Zn} 525 nm; solution yellow). In contrast, the spectrum of the lomofungin-Mg complex showed a bathochromic shift and hyperchromic effect (λ_{max}^{Mg} 495 nm; solution purple). These results clearly indicated that, although they could be formed with the same stoichiometry, the complexes between lomofungin and Mn^{2+} and Zn^{2+} ions were different from each other and also different from those formed with Mg^{2+}.

As several cytotoxic compounds require divalent cations for binding to nucleic acids it was investigated whether the lomofungin-divalent cation complexes could interact with DNA. No evidence could be found in favor of such an interaction. The spectral properties of lomofungin or of the lomofungin-divalent cation complexes were not altered in the presence of native or denatured calf-thymus DNA (results not shown).

Influence of pH on Chelation. Since lomofungin interacts with a divalent cation, it was interesting to titrate the molecule. The spectrum of lomofungin was recorded at different pH values. In Figure 9 can be seen three isosbestic points corresponding to an equilibrium between the neutral and acid forms of the molecule. Three other isosbestic points were also found at basic pH, corresponding to the equilibrium between the neutral and deprotonated forms. Two apparent pK values were obtained, pK_1 = 6.4 ± 0.05 and pK_2 = 11 ± 0.1 (Figure 10).

Assuming that chelation occurs, as in the case of the related compound 8-hydroxyquinoline (Fraser and Creanor, 1974), through one nitrogen atom and the proximal OH group, it was interesting to know whether the deprotonation of the phenolic groups was required for chelation. Practically the same characteristic spectra (λ_{max}) of the complexes were observed at pH 5.8 as at pH 8.0 although the complex

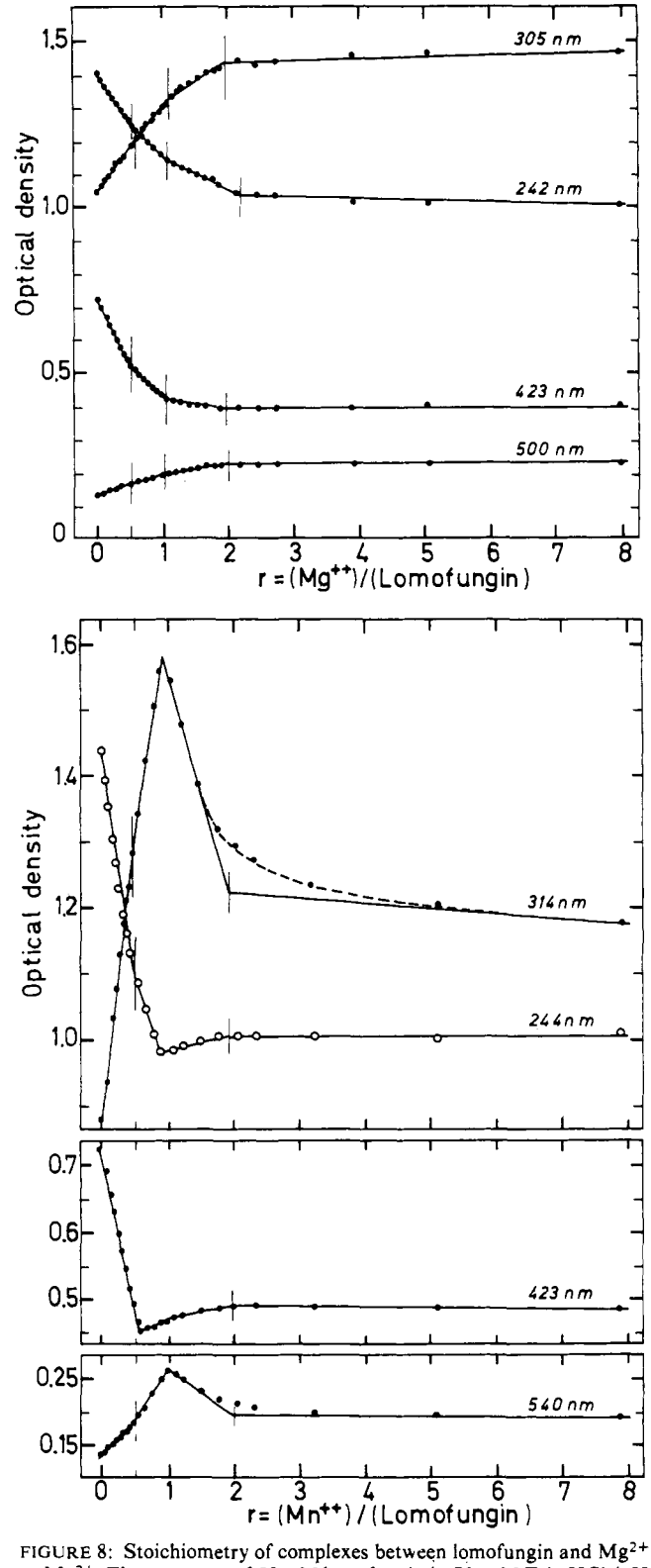

FIGURE 8: Stoichiometry of complexes between lomofungin and Mg^{2+} or Mn^{2+}. The spectrum of 58 μM lomofungin in 70 mM Tris-HCl (pH 8.0) was recorded in the presence of varying concentrations of Mg^{2+} or Mn^{2+} as described under Materials and Methods. Variation of the optical density is shown at four characteristic wavelengths in each case.

(r = 2) with Mg^{2+} appeared somewhat less distinct than with Mn^{2+} or Zn^{2+}. Since the pK values of the three phenolic groups of lomofungin are expected to be around pH 11 (pK_2), the phenolic groups are fully protonated at pH 5.8. The conclusion therefore is that chelation can occur under conditions where the phenolic group is protonated. Al-

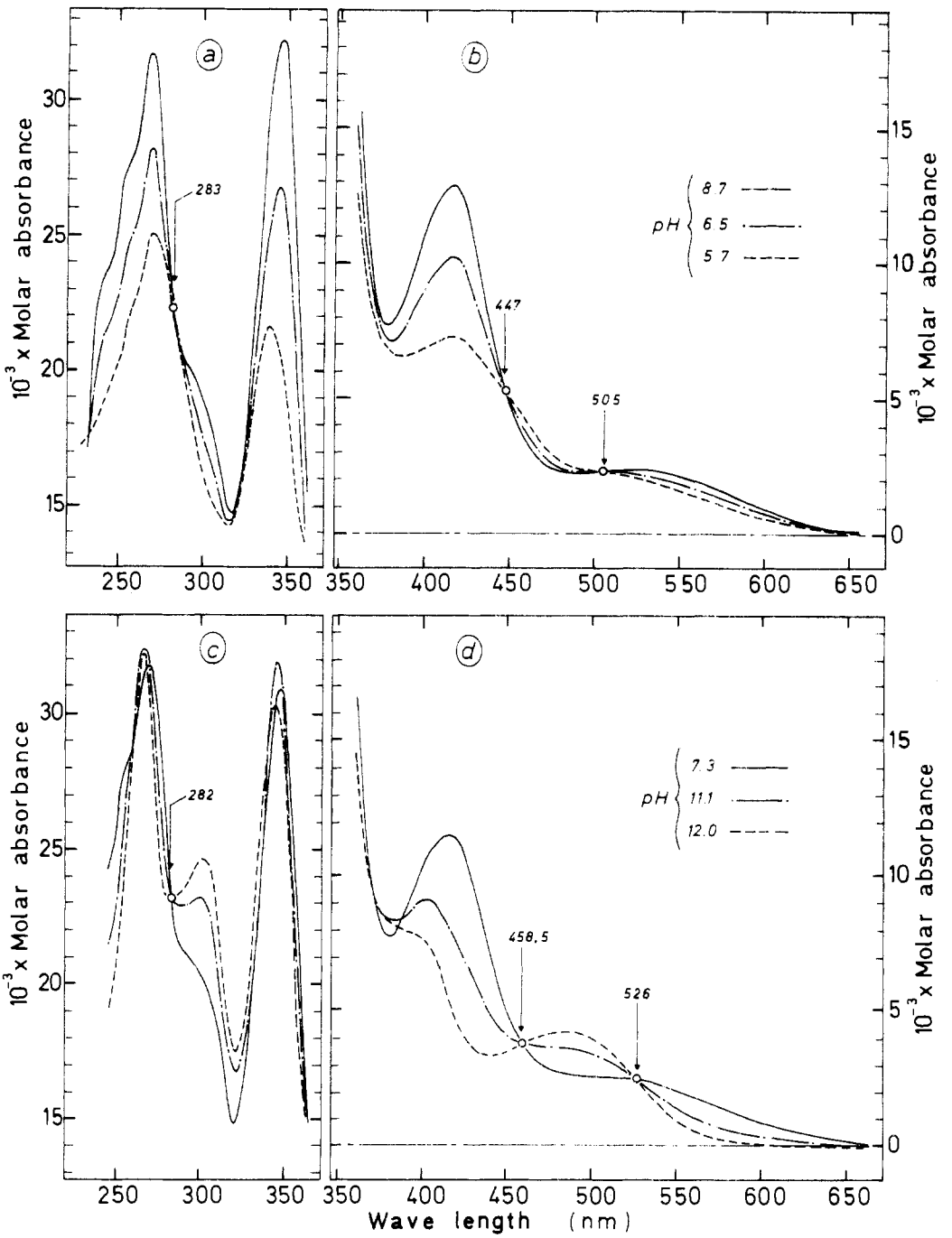

FIGURE 9: Effect of pH on the spectrum of lomofungin. (a and b) The spectrum of 60 μM lomofungin dissolved in 3 ml of water was recorded at varying pH values after the successive addition of microvolumes of 0.01 N HCl (final dilution, 3%). The pH of the initial solution had been adjusted to 9.5 with NaOH. (c and d) Variation of pH in the basic range was achieved similarly by addition of microvolumes of NaOH solutions of increasing concentration (from 0.02 to 6 N NaOH).

though other possibilities cannot be discarded, the above hypothesis would explain the stoichiometry of two cations bound per lomofungin. It should be noted that the complex of stoichiometry 1 cation per dye could also reflect the formation of a polychelate. Such a polymerization process has not been observed but cannot be excluded. Studies on metal chelate compounds of other hydroxyphenazine derivatives, recently reported (Kidani et al., 1975), are in agreement with our observations.

Discussion

The toxic pigment lomofungin affects primarily RNA synthesis in yeast cells and is also a good inhibitor of transcription in vitro. Addition of lomofungin before the start of transcription leads to a reduction in the number of chains initiated, but the drug can also block RNA chain elongation when added during the course of transcription. Lomofungin can therefore block the transcription complex at the initiation as well as the elongation step. In this respect, lomofungin inhibits in vitro transcription in much the same way as luteoskyrin does (Ruet et al., 1973). The extent of inhibition by lomofungin was found to be strongly dependent upon the nature and concentration of the divalent cations used to stimulate transcription. Spectrophotometric studies revealed the formation of different complexes between lomofungin and the divalent cations Mn^{2+}, Mg^{2+}, or Zn^{2+}.

Three types of complexes were found, of respective stoichiometries of 0.5, 1, and 2 metal ions per dye molecule. The chelating properties of lomofungin for Mn^{2+} and Mg^{2+} were also recognized by Fraser and Creanor (1975).

The complete inhibition of transcription observed at low Mn^{2+} concentration probably corresponds to this chelation effect. It should be noted, however, that comparatively little inhibition was observed when Mg^{2+} was used, instead of Mn^{2+}, for transcription by yeast RNA polymerase A or *E. coli* RNA polymerase. It is likely that lomofungin binds Mn^{2+} differently and more strongly than Mg^{2+}. Actually, in many respects the spectra of the complexes formed with Mn^{2+} were different from those obtained with Mg^{2+}. In addition to this primary chelation effect, there remained a secondary inhibition, which was not solely the result of the chelating properties of lomofungin since it was not reversed by addition of an excess of divalent cation. The result suggested a possible interaction between lomofungin, or a lomofungin–Mn complex, with one component of the transcription system. Most of this work was carried out with a 10- to 20-fold molar excess of Mn^{2+} over lomofungin, well above the threshold of chelation; therefore, only this secondary effect was investigated. Several drugs, such as luteoskyrin (Ohba and Fromageot, 1967, 1968), kanchanomycin (Friedman et al., 1969; Joel et al., 1970), as well as olivomycin, chromomycin, and mithramycin (Ward et al., 1965), are known to interact with nucleic acids only in the presence of divalent cations. In the case of lomofungin, however, no evidence was found in favor of such an interaction.

Evidence is presented that lomofungin interacts with RNA polymerase. The interaction process, which was mainly observed with yeast RNA polymerase A, appears to be of two different types. In the presence of Mn^{2+} ions, several molecules of lomofungin bind to the enzyme. The ligand is the lomofungin–Mn^{2+} complex. In addition, even in the absence of divalent cation, lomofungin also favors the dissociation of RNA polymerase A into RNA polymerase A*, which is lacking two polypeptide chains (Huet et al., 1975). Since the same effect can be obtained with dithizone, a zinc chelating agent, it is possible that ZN^{2+} ions are involved in the enzyme structure. In this respect it has been suggested that lomofungin could inhibit transcription by chelating with Zn^{2+} ions bound to the bacterial RNA polymerase (Pavletich et al., 1974). This possibility cannot be excluded although it does not explain why a much stronger inhibition of *E. coli* RNA polymerase was obtained when Mn^{2+} was used to stimulate transcription instead of Mg^{2+}. It is more likely that, when transcription is carried out in an excess of Mn^{2+} ions over lomofungin, the true inhibitory compound is the lomofungin–Mn chelate, which acts at the level of the transcription complex, blocking RNA chain initiation and elongation.

The relevance of the observed interaction of lomofungin with divalent cations as well as with RNA polymerase A to the in vivo effect of the drug remains difficult to assess. The intracellular concentration reached by lomofungin is not known, nor that of divalent cations. Nevertheless, the first interpretation of the antibiotic toxicity rests on its chelating properties. Lomofungin is able to chelate with Mn^{2+} and Zn^{2+} and also to a lesser extent with Mg^{2+}, at pH 5.8, which corresponds roughly to the estimated internal pH of budding yeast (Fraser and Creanor, 1974). The preferential inhibition of synthesis of certain RNA species, particularly of higher molecular weight RNA, rRNA, and polydisperse RNA (Kuo et al., 1973; Fraser et al., 1973), was also observed by Fraser and Creanor (1974) using a different chelating agent, 8-hydroxyquinoline. As this compound binds Mn^{2+} more strongly than Mg^{2+}, they suggested that the effect was due to a different cation requirement from the various RNA polymerases involved. Actually, *E. coli* RNA polymerase or yeast RNA polymerase A is much less inhibited when assayed with Mg^{2+} than with Mn^{2+}. If this interpretation is correct, then it implies that both yeast RNA polymerases A and B require Mn^{2+} for in vivo transcription (Fraser and Creanor, 1975). In fact, whereas RNA polymerase B shows an absolute requirement for Mn^{2+} ions in vitro (Dezelee and Sentenac, 1973), RNA polymerase A is as active with Mg^{2+} as with Mn^{2+} (Buhler et al., 1974). However, the cation requirement of RNA polymerase in vitro might be misleading. A number of anomalous reactions, catalyzed by bacterial RNA polymerase, DNA polymerase, and yeast RNA polymerase, have been described (Dezelee et al., 1974) which only occur in the presence of Mn^{2+}. For this reason it would be important to confirm the specific role of Mn^{2+} ions in DNA transcription in eucaryotic cells. On the other hand, the observation that lomofungin can interact directly with RNA polymerase A and possibly with other proteins indicates that one should not oversimplify the problem by just considering that the drug che-

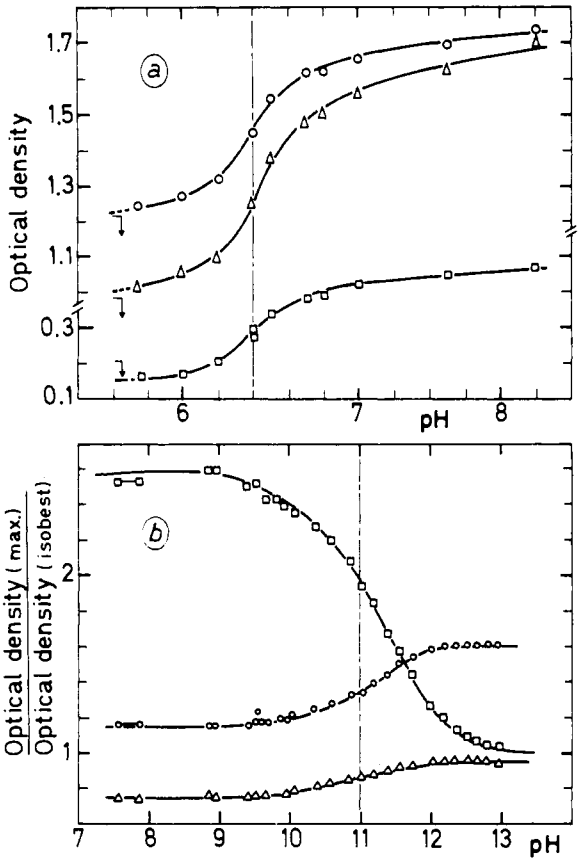

FIGURE 10: Spectrophotometric titration of lomofungin. (a) For measurement of pK_1, the spectrum of 53 μM lomofungin was recorded in 0.1 M potassium phosphate buffer at different pH values (5.8 < pH < 8.0). The optical density is plotted at three wavelengths: (○) 269 nm; (△) 346 nm; (□) 416 nm. (b) Variation of pH was achieved by successive addition of microvolumes of NaOH solutions to a 3-ml solution of 53 μM lomofungin in water (final dilution 7.7%). To disregard the dilution effect for pK_2 measurement the results are given as the ratio of the optical density at a given wavelength vs. the optical density at the nearest isosbestic point. Optical density ratios: (□) at 420 nm vs. 458.5 nm; (○) at 480 nm vs. 526 nm; (△) at 302 nm vs. 282 nm.

lates Mn^{2+} ions in the cell. Actually, at least with yeast cells, lomofungin has a marked killing effect which appears to be correlated with active RNA synthesis. This property allowed the selection of a class of thermosensitive mutants strongly inhibited for RNA synthesis and dominant over the wild type allele (Lacroute et al., 1975). This dominance effect was reminiscent of that of some *E. coli* RNA polymerase mutants. However, using our standard assay procedure, RNA polymerases A and B, isolated from these mutants, did not appear to be more thermosensitive than the enzymes from wild type cells. Nevertheless, it is likely that such dominant mutation affects a fundamental element of the transcription system.

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