In vivo and in vitro effects of rifampicin and streptolydigin on transcription of Kluyveromyces lactis in the presence of nystatin

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ABSTRACT

Rifampicin and streptolydigin, if used in conjunction with nystatin, depress the growth of Kluyveromyces lactis. The incorporation of labeled leucine into protein is inhibited by nystatin whereas the incorporation of labeled uracil into RNA is inhibited by rifampicin in nystatin-treated cells. In order to study the mechanism of inhibition of RNA synthesis we purified by DEAE-Sephadex column chromatography four forms of RNA polymerase from K.lactis cells. The general properties of these enzymes are similar to those of Saccharomyces cerevisiae and of other eukaryotic RNA polymerases. In particular, enzymes IA, IB and III are more active with poly[d(A-T)] template and Mn$^{2+}$ than with native or denatured calf thymus DNA. Enzyme II shows optimal activity with denatured calf thymus DNA and Mn$^{2+}$. When challenged with native calf thymus DNA all enzymes prefer Mg$^{2+}$ as a divalent cation whereas with denatured calf thymus DNA all enzymes are more active with Mn$^{2+}$. Enzyme II is inhibited by α-amanitin but no enzyme is sensitive to rifampicin and streptolydigin. The inhibition of growth and uracil uptake observed when rifampicin is added to nystatin treated cells is probably not caused by a specific inhibition of transcription.

INTRODUCTION

Inhibitors of genetic transcription interacting directly with DNA dependent RNA polymerases are usually restricted in their specificity to either prokaryotic or eukaryotic organisms. This is true for α-amanitin, a mushroom toxin that specifically inhibits a nucleoplasmic RNA polymerase$^1$, and for rifampicin that specifically inhibits prokaryotic RNA polymerases$^2$. Streptolydigin instead, an antibiotic produced by cultures of Strep-
tomyces lydicus, is active on RNA polymerases from bacterial origin and, though only at high doses, on mammalian RNA polymerases. The targets of rifampicin and streptolydigin are very similar. In different bacteria, mutations conferring resistance to rifampicin and streptolydigin map very close and experiments on reconstitution of RNA polymerase from isolated subunits from sensitive and resistant strains showed that both drugs exert their action on the $\beta$-subunit of the enzyme.

On the other hand, the mode of action is different: rifampicin inhibits a particular step in initiation of transcription while streptolydigin inhibits all steps of transcription involving phosphodiester bond formation.

For their specificity of action, rifampicin, streptolydigin and $\alpha$-amanitin are useful tools for in vitro studies of transcription. In vivo studies are, on the contrary, made difficult by the lack of permeability presented by many organisms to these drugs. On yeasts, for instance, rifampicin and streptolydigin are without effect. However, a recent report by Medoff et al. showed that rifampicin affects the growth of Saccharomyces cerevisiae if used in conjunction with amphotericin, a polyene antibiotic active on cell membrane and affecting cell permeability, at concentrations at which either drug is without effect. This raises the possibility that yeast cells contain a biochemical target sensitive to inhibitors specific for prokaryotic RNA polymerases. In order to investigate this possibility we have studied the effect of rifampicin and streptolydigin in combination with nystatin, a polyene antibiotic similar in structure to amphotericin, on Kluyveromyces lactis. Like most species of this genus and in contrast to other yeasts and eukaryotes, K. lactis is insensitive to cycloheximide; we wondered whether also at the level of transcription this organism shows peculiar patterns of sensitivity to inhibitors.
MATERIALS AND METHODS

Yeast Strains, Media and Cell Growth - K.lactis strain 2359 CBS prototroph, was used in all experiments. For RNA polymerase purification cells were grown at 28° in a medium containing 0.5% yeast autolysate (Costantino Corp., Torino, Italy), 1% bacteriological peptone (Oxoid div., Oxo Ltd., London, England), 1% glucose (YEDP) and harvested at a density of 5x10^7 cells/ml, corresponding to a mid-log phase. Minimal medium 40 (ref.16) was used in all experiments with labeled precursors of macromolecules. The effect of the different inhibitor concentrations on cell growth was determined by inoculating 10^5 cells/ml in Minimal medium 40 containing glucose or glycerol as the carbon source and the indicated drug concentrations; the incubation was continued for 34 hours at 28° and the cells counted in a Börker counter.

Substrates - Unlabeled nucleoside triphosphate were purchased from Boehringer Corp., Mannheim, Germany. 3^3-H UTP (1 Ci/mmol) and 1-^14C L-Leucine (50 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England. 2-^14C Uracil (10 mCi/ mmol) from NEN Chemicals GmbH, Frankfurt/Main, Germany.

Chemicals and solutions - Rifampicin, a gift of Dr. G. Iancini, Gruppo Lepetit, Milan, Italy, was dissolved in ethanol. Streptolydigin was a gift of Dr. G.B. Whitfield, Upjohn Corp., Kalamazoo, Mich., USA. The free acid was dissolved in 0.1 M Tris-HCl pH 7.9.

Nystatin was a gift of Pierrel Corp., Milan, Italy, and dissolved in 50% ethanol. α-amanitin was purchased from Boehringer Corp., Ingelheim, Germany, and dissolved in distilled water. Phenylmethylsulfonylfluoride (PMSF) was purchased from Sigma Chemical Co., St.Louis, Mo., USA, and dissolved in ethanol prior to use. Calf thymus DNA was purified from frozen glands^17. Poly[d(A-T)] was a generous gift of Dr.G.Pedrali-Noy.

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In vivo Labeling of RNA and Proteins - For the measurement of RNA synthesis, $^{14}\text{C}$-labeled and unlabeled uracil were added to the final concentrations of 10 µg/ml and 0.01 µCi/ml. For protein synthesis, 10 µg/ml of leucine and 0.025 µCi/ml of $^{14}\text{C}$-leucine were added. At a density of $1 \times 10^7$ cells/ml the labeled cultures were divided into aliquots and treated with various inhibitors. Growth was continued and 1 ml samples were removed at various times and precipitated with 1 ml of cold 10% TCA.

After standing 60 minutes in the cold, $^{14}\text{C}$-uracil containing samples were collected on glass fibre filters (Whatman GF/C) and washed with 5% TCA containing 10 µg/ml or uracil, 5% TCA and ethanol. $^{14}\text{C}$-leucine samples were boiled 15 minutes, cooled, filtered on GF/C and washed with 5% TCA and ethanol. The samples were dried and counted in a scintillation counter.

Assay for RNA polymerase activity - The standard incubation mixture (115 µl) contained 50 mM Tris-Cl, pH 8.1 at 20°, 2.7 mM MnCl$_2$, 1 mM each of ATP, GTP and CTP, 0.025 mM $^3$H-UTP (140 cpm/pmole), 65 µg/ml of native and denatured calf thymus DNA and 50 µl of enzyme fraction per assay. Incubation was at 30° per 30 minutes. The reaction was terminated by pipetting 100 µl of assay mixture on glass fibre filters (Whatman GF/C) and processing for trichloroacetic acid insolubility as described by Bollum. Radioactivity was determined by scintillation counting. The inhibitors, when present, were added to the incubation mixture in the cold; no particular order of addition was followed.

Separation of multiple RNA polymerase activities by DEAE-Sephadex chromatography - Cells washed once with 50 mM Tris-Cl, pH 7.9, 20 mM MgCl$_2$, 0.1 mM EDTA and 100 mM (NH$_4$)$_2$SO$_4$ were resuspended in 4 volumes of 50 mM Tris-Cl, pH 7.9, 20 mM MgCl$_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 200 µg/ml of phenylmethylsulfonylfluoride (PMSF), 800 mM (NH$_4$)$_2$SO$_4$, 20% glycerol and disrupted with glass beads in the cold with a homogenizer. The cell
extract was subjected to disruption in a MSE sonicator with four 30 seconds periods in ice and centrifuged for 4 hr at 36000 rpm in a Spinco 40 rotor at 2°. The supernatant (Fraction I) was subjected to chromatography on DEAE-Sephadex A25 essentially according to Adman et al. 19: 27 ml of Fraction I for a total of 400 O.D. units at 280 nm were diluted to 190 ml with 50 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 1 mM dithiothreitol, 100 µg/ml of PMSF, 20% glycerol (TGEDP buffer) containing 50 mM (NH₄)₂SO₄, and loaded onto a 2.2x13.5 cm DEAE-Sephadex A25 column equilibrated with the same buffer. The column was washed with one volume of 50 mM (NH₄)₂SO₄ in TGEDP buffer and eluted with 10 column volumes of a linear gradient from 50 mM to 400 mM (NH₄)₂SO₄ in TGEDP buffer. The four peaks of activity eluting at 0.17-0.20, 0.21-0.23, 0.27-0.30, 0.35-0.37 M (NH₄)₂SO₄ were pooled and named according to the order of elution, IA, IB, II and III. To each pool glycerol was added to a final concentration of 50%, and the storage was at -20°C. Protein concentration, determined by the procedure of Lowry et al. 20, was 44 µg/ml in pool IA, 33 µg/ml in pool IB, 16 µg/ml in pool II and 17 µg/ml in pool III.

RESULTS

*K. lactis* growth in the presence of inhibitors - Rifampicin and streptolydigin do not affect the growth of *K. lactis* either on solid or liquid media. On the contrary, nystatin produces a sharp decrease in growth rate. In a liquid medium containing glucose as a carbon source (Fig.1) a dose of nystatin of 0.3 µg/ml reduces growth rate to 10% of control value. The addition to various doses of nystatin of 25 µg/ml of rifampicin inhibits growth almost completely. The same effect is observed even at doses of nystatin lower than 0.2 µg/ml at which nystatin alone has no apparent effect on cell growth.
Fig. 1 - Effect of varying concentrations of nystatin on growth in the presence or absence of rifampicin - *K. lactis* was grown to a density of $3.4 \times 10^8$ cells/ml in the medium containing glucose and to $1.8 \times 10^8$ cells/ml in the medium containing glycerol in the absence of inhibitors; for additional details see Materials and Methods. 

- ○○ without rifampicin; △△ with 25 µg/ml of rifampicin.
K. lactis grown in a liquid medium containing glycerol as a carbon source is also sensitive to nystatin and to a combination of nystatin plus rifampicin. The same degree of inhibition is obtained at doses of nystatin lower than those required in a medium containing glucose.

When streptolydigin replaces rifampicin in the mixture with nystatin an inhibition of growth is observed (Table I).

### Table I

Effect of streptolydigin on K. lactis growth.

See Materials and Methods for conditions of growth. Inhibitors, when present, were added at the time of inoculum.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>cells/ml</th>
<th>growth per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$3.6 \times 10^8$</td>
<td>100</td>
</tr>
<tr>
<td>streptolydigin 200 µg/ml</td>
<td>$3.7 \times 10^8$</td>
<td>102</td>
</tr>
<tr>
<td>nystatin 0.2 µg/ml</td>
<td>$3.2 \times 10^8$</td>
<td>92</td>
</tr>
<tr>
<td>nystatin 0.3 µg/ml</td>
<td>$3.3 \times 10^8$</td>
<td>92</td>
</tr>
<tr>
<td>nystatin 0.2 µg/ml streptolydigin 200 µg/ml</td>
<td>$2.8 \times 10^8$</td>
<td>77</td>
</tr>
<tr>
<td>nystatin 0.3 µg/ml streptolydigin 200 µg/ml</td>
<td>$1.7 \times 10^8$</td>
<td>47</td>
</tr>
</tbody>
</table>

At 200 µg/ml of streptolydigin reduction in growth rate is about 50%. In comparison with rifampicin, streptolydigin is active at 10 times higher doses. This is not completely unexpected because streptolydigin is less active than rifampicin also in bacteria.

Synthesis of RNA and proteins in the presence of inhibitors - In order to understand the effect of nystatin and rifampicin on macromolecular syntheses we studied the incorporation of labeled
Fig. 2 - Effect of different inhibitor combinations on the rates of incorporation of $^3$H-uracil or $^{14}$C-leucine into trichloroacetic acid insoluble material. The inhibitors were added at the zero time of the experiment to exponentially growing cultures in the presence of label (for details see Materials and Methods). ○○ without drug; △△ with 0.3 μg/ml of nystatin; □□ with 0.3 μg/ml of nystatin and 25 μg/ml of rifampicin.
precursors into RNA and proteins. The incorporation of \(^{14}\text{C}-\text{uracil}\) into RNA of an exponentially growing culture is not affected, at least for 120 minutes, by the addition of 0.3 \(\mu g/ml\) of nystatin (see Fig.2). However the addition to nystatin of 25 \(\mu g\) of rifampicin causes almost complete inhibition of incorporation.

On the contrary, in a parallel culture labeled with \(^{14}\text{C}-\text{leucine}\) the incorporation of label into protein is arrested by 0.3 \(\mu g/ml\) of nystatin. The addition of rifampicin does not change the time or extent of protein synthesis inhibition caused by nystatin. Thus, RNA synthesis is not arrested by nystatin in this organism whereas protein synthesis is. The same result as shown in Fig.2 for a dose of nystatin of 0.3 \(\mu g/ml\) is also observed at lower doses (results not shown). Synthesis of protein is always inhibited by nystatin while uracil incorporation is stopped by the mixture of nystatin and rifampicin.

Properties of RNA polymerases - In order to investigate the nature of the putative target of rifampicin and streptolydigin inhibition of growth and to explain the observed reduction of RNA synthesis (Fig.2) we purified the RNA polymerases from exponentially growing cells. Four peaks of activity (see Fig.3) were eluted from a DEAE-Sephadex column (see Materials and Methods for details of purification procedure). The designations IA, IB, II and III to the peaks in order of elution from the column was established after comparison with a parallel purification of the enzymes from \(S.\text{cerevisiae}\) according the procedure described by Adman et al.\(^{19}\) (results not shown).

Like for \(S.\text{cerevisiae}\) enzymes the recovery of activity in peak IA varies greatly from one preparation to the other. Though protein concentration in peak IA is higher than in IB, this enzyme is the less stable one. Peak IB is more abundant in activity than peak IA, though no large differences are observed in template specificity and divalent ions activation (Ta-
Fig. 3 - DEAE-Sephadex column chromatography of RNA polymerase - (For details of purification procedure see Materials and Methods). According to the order of elution (from left to right) the peaks were named enzyme IA, IB, II and III.
TABLE II
RNA polymerase activities as a function of template.
Activities are expressed as nmol of $^3$H UTP incorporated $\times$ hour$^{-1}$ $\times$ mg$^{-1}$. The assay conditions were as described in Materials and Methods with the following specifications: native, denatured calf thymus DNA and poly[d(A-T)] were 32 µg/ml of assay. With poly[d(A-T)] template only two triphosphates were present: 0.025 mM $^3$H UTP and 2.5 mM ATP. 2.7 mM Mn$^{2+}$ was always present.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>IA</th>
<th>IB</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>native calf thymus DNA</td>
<td>0.2</td>
<td>0.1</td>
<td>1.9</td>
<td>1.6</td>
</tr>
<tr>
<td>denatured calf thymus DNA</td>
<td>0.1</td>
<td>0.1</td>
<td>8.8</td>
<td>4.9</td>
</tr>
<tr>
<td>poly[d(A-T)]</td>
<td>0.5</td>
<td>0.6</td>
<td>2.7</td>
<td>31.7</td>
</tr>
</tbody>
</table>

TABLE III
Ratios of RNA polymerase activities as a function of DNA and divalent ions.
RNA polymerase assay contained the usual reagents as described in Materials and Methods, except that 5 mM MgCl$_2$ was present where indicated. Divalent ions are at optimal concentrations. Ammonium sulfate was 50 mM with enzyme IA, 65 mM with enzyme IB, 80 mM with enzyme II and 280 mM with enzyme III. Calf thymus DNA, native or denatured, was 65 µg/ml.

<table>
<thead>
<tr>
<th></th>
<th>nat.DNA/den.DNA activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>Mg$^{2+}$</td>
</tr>
<tr>
<td>Enzyme IA</td>
<td>2</td>
</tr>
<tr>
<td>Enzyme IB</td>
<td>4</td>
</tr>
<tr>
<td>Enzyme II</td>
<td>0.7</td>
</tr>
<tr>
<td>Enzyme III</td>
<td>-</td>
</tr>
</tbody>
</table>
Peak II is easily identified as the enzyme sensitive to inhibition by α-amanitin (Fig.4). Peak III is the enzyme most active with poly[d(AT)] template (Table II), prefers denatured over native DNA, at low salt concentration, and Mn$^{2+}$ as the divalent ion. The efficacy of native and denatured DNA and divalent ions in stimulating RNA synthesis is shown in Table III. Enzymes IA and IB prefer native over denatured DNA and Mg$^{2+}$ as a divalent ion. When denatured DNA is used, Mn$^{2+}$ is the preferred ion by all enzymes. Enzyme II prefers denatured over native DNA and Mn$^{2+}$.

The activity of enzyme III is highly dependent on salt concentration; at high concentration (Table III) native DNA is a better template, whereas, as indicated above, the opposite is true at low salt concentration. Enzyme IA, IB and II show maximal activity below 0.1 M (NH$_4$)$_2$SO$_4$ while enzyme III is active in a broad range of salt concentrations (between 0.1 and 0.3 M (NH$_4$)$_2$SO$_4$).

Effect of inhibitors of RNA polymerase - The sensitivity of RNA polymerase towards rifampicin, streptolydigin, nystatin and mixtures of them was tested through all steps of the purification procedure. No fraction was found sensitive to these drugs. Figure 4 shows the sensitivity of the four purified RNA polymerases towards α-amanitin, rifampicin and streptolydigin. Enzyme II is inhibited almost completely (95%) by 10 µg/ml of α-amanitin while enzyme IA, IB and III are unaffected even by high concentrations of drug. The results are very similar to those obtained with *S. cerevisiae* RNA polymerases. No inhibition is observed with doses of streptolydigin and rifampicin as high as 200 µg/ml on all four enzymes. The lack of inhibition by rifampicin has been already observed with mammalian RNA polymerases and with *S. cerevisiae* enzymes. The lack of inhibition by 200 µg/ml of streptolydigin on all polymerases distinguishes yeast polymerases from the mammalian ones. L-120 cells RNA polymerase, is more than 50% inhibited by 50 µg/ml of strepto-
Fig. 4 - Inhibition of RNA polymerase IA, IB, II and III by α-amanitin, rifampicin or streptolydigin - The assay (see Materials and Methods) contained denatured and native calf thymus DNA. The amount of UTP polymerized was 10 pmol for enzyme IA and IB, 50 pmol for enzyme II and 42 pmol for enzyme III. ▲▲ enzyme IA; ■■ enzyme IB; ●● enzyme II; ▼▼ enzyme III.
lydigin⁵. Though higher doses are required, calf thymus RNA polymerases A and B are also inhibited⁶.

It should be noticed, however, that variations in activity, among different batches of streptolydigin may complicate the comparison between results obtained in different laboratories when high doses of drug are employed.

DISCUSSION

The addition of rifampicin to K.lactis cultures growing at different rates because of the presence of various concentrations of nystatin results in a further decrease of growth rate. Similar results are obtained by the combination of streptolydigin and nystatin. Since rifampicin and streptolydigin are specific inhibitors of transcription these results could be explained by their interaction with the transcription machinery of yeast cells. In order to define the nature of the biochemical target of rifampicin we have measured the incorporation of labeled precursors of RNA and proteins in the presence of nystatin alone and in combination with rifampicin. As seen in Fig. 2 the effect of nystatin at a dose that reduces growth to 10% of the control is essentially only on protein synthesis whereas the effect of rifampicin is, on cells in which protein synthesis is already blocked by nystatin, on RNA synthesis.

Since nystatin is not a specific inhibitor of protein synthesis, the reduction of incorporation of label into protein, observed in vivo, may be caused by either a reduction of leucine uptake or a general leakage of aminoacids from these cells. However, the reduction of incorporation of uracil into RNA caused by the addition of rifampicin, could result from a direct inhibition of transcription. In order to verify this hypothesis we have isolated four chromatographically distinct RNA polymerases and assayed them in the presence of rifampicin and
streptolydigin. One enzyme only is sensitive to α-amanitin while none is sensitive to rifampicin or streptolydigin. This latter result argues that the effect of rifampicin on growth and on uracil uptake is not due to inhibition of RNA polymerase. Since none of the purified enzymes is of mitochondrial origin, the possibility that mitochondrial RNA polymerase is the target of rifampicin and streptolydigin cannot be ruled out. Recent work on purification and characterization of mitochondrial RNA polymerase showed that the enzyme from Xenopus laevis ovaries is rifampicin insensitive, whereas the enzymes from rat liver, Neurospora crassa and Blastocladiella emersonii are inhibited by the drug; for S. cerevisiae there are two conflicting reports showing respectively sensitivity and insensitivity to rifampicin. We have not attempted to purify the mitochondrial RNA polymerase from K. lactis and we do not have evidence on the sensitivity of mitochondria towards rifampicin. However a dose-response curve of growth in a medium containing glycerol as a carbon source shows that K. lactis is no more sensitive to rifampicin than when grown on a medium containing glucose (Fig.1). The failure to observe an increase in the inhibition caused by rifampicin in condition of growth that depends on mitochondria integrity, suggests that mitochondrial transcription is probably not the function inhibited by rifampicin. All these results argue that rifampicin and streptolydigin when used in conjunction with nystatin do not affect transcription specifically but probably enhance the effect of nystatin on membranes.

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