Inhibition by Derivatives of Rifamycin of Soluble Ribonucleic Acid Polymerase from Rat Liver

By TOSHIO ONISHI and MASAMI MURAMATSU

Department of Biochemistry, Tokushima University School of Medicine, Tokushima, Japan

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Multiple forms of DNA-dependent RNA polymerase (EC 2.7.7.6) have been demonstrated in eukaryotic cells (Roeder & Rutter, 1969; Jacob *et al.*, 1970a; Kedinger *et al.*, 1970; Mondal *et al.*, 1970; Horgen & Griffin, 1971; Strain *et al.*, 1971). RNA polymerase I (or A), which is associated with the nucleolus (Roeder & Rutter, 1970), is probably engaged in the transcription of nucleolar genes to form rRNA (Blatti *et al.*, 1970; Tocchini-Valentini & Crippa, 1970; Zylber & Penman, 1971), whereas RNA polymerase II (or B), which is present in the nucleoplasmic fraction (Roeder & Rutter, 1970a), is probably making extranucleolar nuclear RNA, including heterogeneous nRNA and mRNA (Blatti *et al.*, 1970; Tocchini-Valentini & Crippa, 1970).

Selective inhibitors of RNA synthesis catalysed by these enzymes and of each step of the RNA polymerase reaction would be useful in exploring the mechanism of RNA synthesis in nucleated cells.

α -Amanitin is a potent and specific inhibitor of nucleoplasmic RNA polymerase (Jacob *et al.*, 1970a; Kedinger *et al.*, 1970; Blatti *et al.*, 1970; Lindel *et al.*, 1970), affecting the elongation step (Kedinger *et al.*, 1970; Lindel *et al.*, 1970; Jacob *et al.*, 1970b).

The activity of mitochondrial RNA polymerase is selectively inhibited by rifampicin and rifamycin (Shmerling, 1969; Reid & Parsons, 1971), which are inhibitors of prokaryotic RNA polymerase (Hartmann *et al.*, 1967; Wehrli *et al.*, 1968) at the initiation step (Wehrli *et al.*, 1968; Sippel & Hartmann, 1968). Also, Butterworth *et al.* (1971) have reported the effect of a rifamycin analogue on the transcription of mammalian chromatin by mammalian DNA-dependent RNA polymerase.

Horgen & Griffin (1971) reported that cycloheximide inhibited specifically nucleolar RNA polymerase (polymerase I) of *Blastocladiella emersonii*. However, the activity of RNA polymerase solubilized from the nucleoli of rat liver was not affected by cycloheximide even at a concentration as high as 1 mg/ml (Higashinakagawa & Muramatsu, 1972).

In the course of screening drugs that may affect RNA polymerase we have investigated several rifamycin derivatives including rifamide, the *O*-octyloxime of 3-formylrifamycin SV (compound AF/O13) and 4-*N*-benzyl-2,5-dimethyldemethylrifampicin (compound AF/ABDMP). The present paper shows that compounds AF/ABDMP and AF/O13 inhibit

the soluble DNA-dependent RNA polymerases I and II of rat liver. These drugs seem to inhibit the RNA polymerase activities at a step prior to polymerization of ribonucleotides, possibly as a result of interaction with the RNA polymerase molecules.

Materials and methods

Rifampicin, rifamide and compounds AF/ABDMP and AF/O13 were kindly supplied by Lepetit Pharmaceuticals Ltd. (Maidenhead, Berks., U.K.). [14 C]UTP (50 mCi/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.).

The nucleolar and extranucleolar nuclear fractions were obtained by a modification (Higashinakagawa *et al.*, 1972) of the sonication procedure described by Muramatsu (1970), with Mg^{2+} instead of Ca^{2+} . The nucleolar and extranucleolar nuclear RNA polymerases were purified from these fractions by $(NH_4)_2SO_4$ precipitation followed by column chromatography on DEAE-Sephadex A-25 by the procedure described by Roeder & Rutter (1969, 1970). RNA polymerases I and II were each eluted as single peaks, at 0.15 M- and 0.25 M- $(NH_4)_2SO_4$ respectively. The eluate was stored at $-70^\circ C$ after the addition of bovine serum albumin (final concn. 1 mg/ml). The standard reaction mixture for the assay of RNA polymerase activity contained, in 0.15 ml: 5 μ mol of tris-HCl buffer (pH 7.9 at $37^\circ C$), 0.05 μ mol of dithiothreitol, 0.24 μ mol of $MnCl_2$, 0.4 μ mol of ATP, 0.1 μ mol each of GTP and CTP, 20 μ g of native calf thymus DNA (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), 0.1 μ Ci of [14 C]UTP, various concentrations of drugs and 15–20 units of RNA polymerase in solution containing bovine serum albumin (1 mg/ml). (A unit of RNA polymerase is defined as the amount causing the incorporation of 1 pmol of [14 C]UMP into acid-insoluble material in 10 min at $37^\circ C$.) The final salt concentrations of the reaction mixture were 0.03 M and 0.1 M with respect to $(NH_4)_2SO_4$ for RNA polymerases I and II respectively. Mn^{2+} was used throughout, since even RNA polymerase I was more active with this ion than with Mg^{2+} (Roeder & Rutter, 1969).

After incubation of the mixture for 10 min at $37^\circ C$, a 0.1 ml portion was delivered on to a glass-fibre disc (Whatman GF/C), which was then immersed in ice-cold 5% (w/v) trichloroacetic acid–1% (w/v)

sodium pyrophosphate for 15 min. The discs were washed four times by resuspension in the same medium (5 min each time) and finally once by immersion in ethanol for 10 min. Radioactivity was determined in toluene scintillation fluid in a Packard Tri-Carb liquid-scintillation spectrometer.

Results and discussion

In agreement with observations with calf thymus RNA polymerases (Chambon *et al.*, 1970), rifampicin had little inhibitory effect on the activity of either of the two enzymes from rat liver, even at a concentration of 500 $\mu\text{g}/\text{ml}$ (Fig. 1a). Rifamide at 500 $\mu\text{g}/\text{ml}$ inhibited each RNA polymerase activity by only 20% (Fig. 1b). Although compounds AF/ABDMP and AF/O13 were more potent as inhibitors of RNA synthesis, they inhibited both RNA polymerase activities to almost the same extents (Figs. 1c and 1d); compound AF/O13 was more effective than compound AF/ABDMP, showing 95% inhibition at a concentration of 50 $\mu\text{g}/\text{ml}$, whereas 300 μg of compound AF/ABDMP/ml was required for the same effect. To analyse the mode of action of the latter two drugs on transcription by RNA polymerases I and II, [^{14}C]UTP and one of the drugs were added either at the start of incubation or after the reaction had progressed for 5 min at 37°C. As shown in Figs.

2(c) and 2(d), transcription by RNA polymerase II was not inhibited by compound AF/O13 or by compound AF/ABDMP when either was added to the reaction mixture after RNA synthesis was already initiated. These results indicate that these drugs inhibit RNA synthesis at a step before elongation. With RNA polymerase I, however, partial inhibition was noted when the drugs were added to the assay mixture even after RNA synthesis was initiated (Figs. 2a and 2b). This partial and progressive inhibition may be explained in either one of two ways, under the assumption that these drugs inhibit initiation only: (1) RNA polymerase I may be able to initiate RNA synthesis at restricted regions on the DNA template, so that re-initiation occurs more frequently in this system than with RNA polymerase II; (2) with RNA polymerase I formation of the enzyme-DNA complex or initiation might take place more slowly than with RNA polymerase II in our system. Another explanation, namely that only RNA polymerase I could be influenced partially at the elongation step, cannot be completely ruled out, although it is not very likely.

Actinomycin D, which binds with DNA and inhibits RNA synthesis mostly at the step of elongation, was in fact effective irrespective of the time of addition of the drug (Fig. 2a).

Further experiments to determine the binding of

Fig. 1. Effect of rifamycin derivatives on soluble DNA-dependent RNA polymerase activities from rat liver

Experimental details are as described under 'Materials and methods'. Drugs were dissolved in dimethyl sulphoxide. In the control, the assay was carried out in the presence of the same final concentration of dimethyl sulphoxide (6.6%, v/v) instead of the drug. ○, RNA polymerase I; ●, RNA polymerase II.

Fig. 2. Inhibition of RNA polymerase activities by compounds AF/ABDMP and AF/O13 added at different times during incubation

Reaction mixtures scaled up fourfold from that described under 'Materials and methods' were employed. ●, After preincubation for 5 min (at 37°C) of the reaction medium (0.54 ml) containing the reactants except for $[^{14}\text{C}]\text{UTP}$ and the drug, 0.04 ml of dimethyl sulphoxide and 0.02 ml of $[^{14}\text{C}]\text{UTP}$ were added to the incubation mixture. ▲, To the preincubated mixture described above were added $[^{14}\text{C}]\text{UTP}$ and 0.04 ml of compound AF/ABDMP (3 mg/ml) (b and d) or compound AF/O13 (600 $\mu\text{g}/\text{ml}$) (a and c). ○, $[^{14}\text{C}]\text{UTP}$ and the drug were added at the start of the incubation. ▼, Actinomycin D (final concn. 5 $\mu\text{g}/\text{ml}$) was added instead of compound AF/ABDMP or compound AF/O13, after preincubation for 5 min. ▽, Actinomycin D was added at the start of the incubation. At intervals during incubation at 37°C, 0.1 ml portions were removed and the radioactivity was determined as described under 'Materials and methods'.

these drugs with RNA polymerase I or II were inconclusive because of the non-specific interaction of albumin with these drugs. Radioactively labelled drugs may be needed to clarify these points.

Gurgo *et al.* (1971) reported that compound AF/ABDMP inhibited the RNA-dependent DNA polymerase of some oncogenic RNA viruses at a concentration of 100 µg/ml, whereas DNA-dependent DNA polymerases from KB cells, *Escherichia coli* or mouse sarcoma virus were not affected appreciably at the same concentration.

Crippa & Tocchini-Valentini (1971) and Ficq & Brachet (1971) have suggested a possible role of RNA-dependent DNA polymerase in the amplification of ribosomal genes during the early oogenesis of *Xenopus laevis*. Their conclusion is at least partly based on the specificity of the action of compound AF/ABDMP on this enzyme. It is particularly noteworthy that transcription in both directions (DNA→RNA and RNA→DNA) is inhibited by the same drug, compound AF/ABDMP, with respect to ribosomal genes. This may help to shed some light on the mechanism of these apparently opposite but related reactions.

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