RNA POLYMERASE B FROM AN α-AMANITIN RESISTANT MOUSE MYELOMA CELL LINE

Elisabeth WULF and Linda BAUTZ

Universität Heidelberg, Lehrstuhl für Molekulare Genetik, Im Neuenheimer Feld 230, 6900 Heidelberg, Germany

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1. Introduction

Mutant cell lines resistant to the cytotoxicity of α-amanitin have been established from rat myoblasts [1], Chinese hamster ovary [2,3] and BHK-T6 hamster cells [4]. All these mutant cell lines exhibit an altered form of RNA polymerase B under in vitro conditions which is more resistant to the action of α-amanitin than the RNA polymerase activity isolated from wild type cells.

The finding of Somers et al. [5] that the RNA polymerase activity in the α-amanitin resistant rat myoblast cell line L_6 appears to be both of the sensitive and the resistant form, suggested that resistance to the toxin is a phenotypically dominant trait [1]. In the case of the α-amanitin resistant Chinese hamster ovary (CHO) cells however, the gene coding for the resistance to the toxin seems to be in a hemizygous condition since only resistant enzyme was found in these CHO cells [2].

In this paper we report the isolation and biochemical characterisation of an α-amanitin resistant mutant selected from the mouse myeloma cell line MOPC 104 E. In contrast to the α-amanitin resistant mutants described by other investigators which appear to be polyploid for the gene coding for the α-amanitin binding subunit we report here the finding of a mutant cell line which is diploid with regard to the synthesis of resistant and sensitive RNA polymerase B enzyme.

2. Materials and methods

2.1. *Cell culture and mutant selection*

The cell line, MOPC 104 E, derived from a mineral oil-induced myeloma tumor in Balb/c mice (M. Potter, National Institute of Health, Bethesda) was adapted for growth in cell culture and used as a source in selection of the α-amanitin resistant cell line MOPC 104 E Ama.

Cells were routinely grown in suspension culture in modified RPMI-medium (Flow Laboratories) supplemented with 10% foetal calf serum (Gibco) at 37°C in humified incubators with 5% CO_2–95% air.

The isolation of a mutant cell line, resistant to the cytotoxic action of α-amanitin, was obtained by successive exposure of wild type cells to increasing concentrations of the drug (0.5, 1.0, 2.0, 3.0 and 5.0 μg/ml). Mutant cells were selected, which were resistant to 5 μg/ml α-amanitin.

2.2. *Enzyme solubilisation and DEAE-cellulose chromatography*

MOPC 104 E- and MOPC 104 E Ama-RNA polymerase B enzymes were solubilized according to the modified procedure of Somers et al. [5]. For each enzyme extraction $5\text{–}8 \times 10^8$ log phase cells were washed twice with phosphate-buffered saline (pH 7.5) and resuspended at approximately 10^8 cells/ml in 0.05 M Tris-HCl, pH 7.9 (22°C), 1 mM EDTA, 0.01 M thioglycerol (Fluka, Switzerland), 0.1 mM dithio-

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erythritol and 25% (v/v) glycerol (buffer A) containing 5 mM $MgCl_2$ and 1.7 mM phenylmethylsulfonylfluoride (PMSF, Merck). Ammonium sulfate (2 M, pH 7.9) was added to a final concentration of 0.3 M. The cell suspension was sonicated (4 × 20 s, MSE sonicator) and then diluted with 2 volumes of buffer A containing 5 mM $MgCl_2$ and 1.7 mM PMSF. After centrifugation for 45 min at 35 000 rpm in a Beckman 50.1 rotor the chromatin containing pellet was discarded and the supernatant, which had an ammonium sulfate concentration less than 0.1 M, was used directly for chromatography on a 0.9 × 7 cm column of DEAE-cellulose.

DEAE-cellulose (Whatman DE-52) was prepared according to Kedinger et al. [6]. Columns were washed with buffer A containing 0.1 M ammonium sulfate and the A enzyme was eluted with 0.15 M, the B enzyme with 0.3 M ammonium sulfate.

Peak fractions containing the bulk of the eluted RNA polymerase B enzyme were pooled and the sensitivity of the enzyme to inhibition by increasing amounts of α-amanitin was measured under standard assay conditions.

2.3. *Standard RNA polymerase assay*

Standard assay mixtures (0.25 ml) contained 0.08 M tris-HCl, pH 7.9 (22°C), thioglycerol, 0.1 mM dithioerythritol, 1 mM $MnCl_2$, 12% glycerol, 0.05 M ammonium sulfate, 0.5 mM ATP, CTP and GTP, 5 μCi (^{3}H)-UTP (Amersham Buchler), 50 μg denatured calf thymus DNA, the indicated amounts of enzyme (20–50 μl) and α-amanitin at the concentrations noted. After 20 min at 37°C the reactions were terminated and trichloracetic acid insoluble material was collected and washed on GF/A (Whatman) glass fiber filters and the radioactivity counted in toluene-base scintillation fluid [7].

2.4. *Binding of amanitin to RNA polymerase B*

The binding of *O*-[^{3}H]-methyl-demethyl-γ-amanitin by RNA polymerase B was determined using the membrane filter technique as described by Cochet-Meilhac et al. [8,9].

100 μl of the DEAE cellulose purified RNA polymerase B were incubated with increasing concentrations of *O*-[^{3}H]-methyl-demethyl-γ-amanitin (2.4 Ci/mmol) for 12 h at 20°C in 0.25 ml of 0.15 M Tris–HCl, pH 7.9 (22°C), 0.1 mM dithioerythritol and 35% (v/v) glycerol (binding buffer). After incubation the reaction mixture was filtered on nitrocellulose filters. (HAWP millipore filter, diameter 25 mm, pore size 0.45 μm.)

The filters were washed with 0.1 M Tris–HCl, pH 7.9 (22°C), 0.1 mM EDTA, 0.1 mM dithioerythritol, 1.5% (v/v) dimethylsulfoxide (Merck) and 15% (v/v) glycerol (washing buffer) and completely dissolved in 10 ml Bray's scintillation fluid for counting.

Blank values were obtained by preincubating the RNA polymerase B with unlabeled α-amanitin (1 μg/ml) for 60 min at 0°C before addition of labeled amanitin.

3. Results

3.1. *Selection of an α-amanitin resistant MOPC 104 E mutant*

α-Amanitin resistant cells were selected by adaption of the wild type myeloma cell line to successively increasing concentrations of α-amanitin. The cells used in all the experiments were resistant to 5 μg/ml α-amanitin and were called MOPC 104 E Ama in contrast to the wild type cells MOPC 104 E.

Survival rates of MOPC 104 E and MOPC 104 E Ama are shown in fig.1. It can be seen that the rate of survival of the wild type was down to 50% after

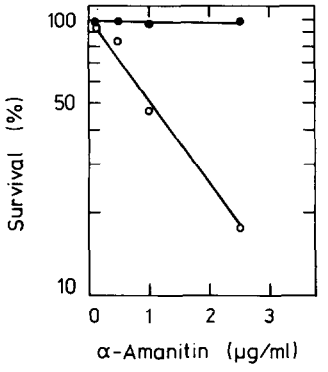

Fig.1. Survival curves for MOPC 104 E and MOPC 104 E Ama. Wild type and mutant cells from exponentially growing cultures were diluted in fresh medium containing increasing concentrations of α-amanitin. After 72 h the percentage of viable cells resistant phenotype was determined using trypan blue staining. ○———○ MOPC 104 E, ●———● MOPC 104 E Ama.

a 3 days exposure to α-amanitin at concentrations of 1 μg/ml whereas the mutant showed no sign of inactivation after several days of exposure to the toxin. It should be noted here that the generation time of MOPC 104 E and mutant MOPC 104 E Ama cells were 20 and 16 h, respectively, and that the resistant phenotype of the mutant cell line was stable for 20–100 generations of growth in the absence of α-amanitin.

3.2. *Isolation and characterisation of RNA polymerase B from MOPC 104 E Ama cells*

In order to do biochemical studies on the α-amanitin resistant mutant cell line, the RNA polymerases from wild type and from mutant cells were partially purified by DEAE-cellulose chromatography. The elution profiles of the RNA polymerases from both cell lines showed two major peaks of activity (data not shown) classified as enzymes A (I) and B (II) because of their elution position and sensitivity to α-amanitin [10–12]. Elution was carried out via a 0.03–0.5 M ammonium sulfate gradient or stepwise. Enzyme A always chromatographed at 0.15 M ammonium sulfate and was resistant to 10 μg/ml α-amanitin in the in vitro assay. The B enzyme always chromatographed at 0.3 M ammonium sulfate and was partially resistant to 0.1 μg/ml amanitin when isolated from mutant cells, while the B enzyme from wild type cells was completely inhibited by the toxin at this concentration. In all experiments described here we used the B enzyme obtained by stepwise elution. The pooled peak fractions of the RNA polymerase B from wild type and mutant cells were subjected to a range of α-amanitin concentrations. The data presented in fig.2 show that about 50% of the Ama-mutant polymerase B activity was resistant to 0.01 μg/ml α-amanitin, while most of the B enzyme activity from wild type cells was inhibited at this α-amanitin concentration. The biphasic inhibition obtained for the Ama-mutant shows that cells resistant to amanitin in vivo and grown for several generations in amanitin-free medium synthesized both the resistant and the sensitive enzyme at nearly 1 : 1 relationship. Mutant cells grown continuously on amanitin medium and those grown only for one generation on medium without α-amanitin showed a higher percentage of resistant enzyme, but no complete resistance of the enzyme was observed. As can be seen in fig.2 about one fourth

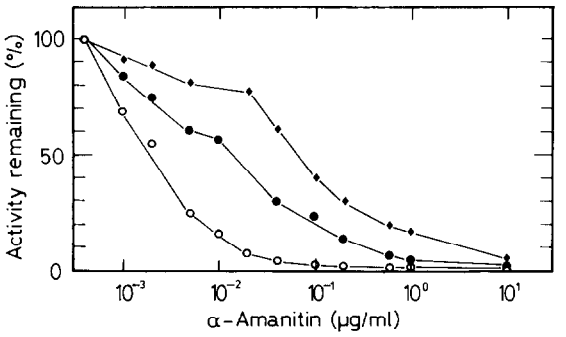

Fig.2. Sensitivity of MOPC 104 E and MOPC 104 E Ama RNA polymerase B to inhibition by α-amanitin. DEAE cellulose purified enzyme B was assayed as described in Materials and methods in the presence of increasing amounts of α-amanitin. The α-amanitin was added to the reaction mixture at 0°C prior to enzyme addition. Every assay was run in duplicate and the results were averaged. o--------o RNA polymerase B (50 μl enzyme solution/assay) from MOPC 104 E cells. •--------• RNA polymerase B (20 μl enzyme solution/assay) from MOPC 104 E Ama cells grown for many generations in the absence of α-amanitin. ♦--------♦ RNA polymerase B (50 μl enzyme solution/assay) from MOPC 104 E Ama cells grown continuously in medium containing α-amanitin (5 μg/ml) and RNA polymerase B (50 μl) from MOPC 104 E Ama cells grown for one generation without α-amanitin.

of the enzyme activity which is found even under conditions where the cells are grown continuously in the presence of the toxin is α-amanitin sensitive.

3.3. *Binding studies of O-[^{3}H]-methyl-demethyl-γ-amanitin to RNA polymerase B molecules*

The binding conditions and membrane filter assay described by Cochet-Meilhac et al. have shown that RNA polymerase B and radioactively labeled amatoxins bind in a 1 : 1 stoichiometry, indicating only one binding site on the polymerase B molecule for amanitin [9]. We have used this technique to determine the amount of sensitive RNA polymerase B molecules present in the amanitin resistant MOPC 104 E Ama mutant, grown with or without the toxin. We were able to show by the same binding technique that an exchange of α-amanitin molecules on the polymerase does take place, since incubation with radioactive amanitin resulted in replacement of the unlabeled amanitin by the [^{3}H]-labeled toxin. Increasing amounts of the radioactive amanitin derivate

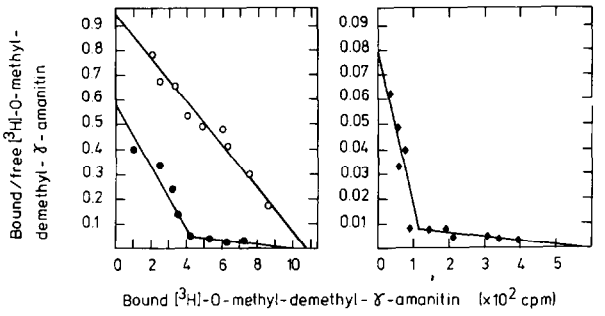

Fig.3. Scatchard plot of *O*-[^{3}H]-methyl-demethyl-γ-amanitin binding to RNA polymerase B. Increasing concentrations of [^{3}H]-amanitin were incubated for 12 h at 20°C with 100 μl DEAE cellulose purified enzymes B of MOPC 104 E (o- - -o); MOPC 104 E Ama, grown in the absence of α-amanitin (•- - -•); and MOPC 104 E Ama, grown for many generations in the presence of 5 μg/ml α-amanitin and for one generation without (♦- - -♦) in a final volume of 0.25 ml binding buffer. The determination of bound amanitin in complexes between *O*-[^{3}H]-methyl-demethyl-γ-amanitin and RNA polymerase B was performed by using the nitrocellulose membrane filter method as described in Materials and methods. Binding data for α-amanitin cells, grown continuously on α-amanitin medium were the same as for mutant cells grown for one generation without the toxin.

were incubated with partially purified RNA polymerase B from the amanitin resistant mutant and from wild type cells, under standard conditions. A scatchard plot of the binding curves is shown in fig.3 for the wild type enzyme, the mutant enzyme from cells grown for several generations without the toxin, and for the enzyme isolated from cells grown for one generation only on amanitin–free medium.

The determination of the equilibrium dissociation constant (Kd) of complexes between *O*-[^{3}H]-methyl-demethyl-γ-amanitin with wild type and with mutant RNA polymerase B gave K_d values of approximately 4×10^{-10} M and 4×10^{-8} M, respectively. The K_d value of 4×10^{-10} M obtained for the wild type enzyme corresponds well with the K_d of 5×10^{-10} M obtained by Cochet-Meilhac for wild type RNA polymerase B from different sources using the nitrocellulose filtration technique which was also used exclusively in our binding experiments [8]. If we compare the differences in K_d values for the two enzymes with the difference in concentration of α-amanitin required to give 50% inhibition, a value of approximately two logs is also observed (fig.2).

The results from the binding experiments correspond well with those obtained from α-amanitin inhibition curves, supporting our finding that about 50% RNA polymerase B in mutant cells grown for several generations without the toxin is sensitive enzyme. In addition, the results from the Scatchard analysis for the enzyme from mutant cells grown for one generation without the toxin and from cells grown continuously on the drug (data not shown) suggest that even in the latter cases we find a coexpression of sensitive and resistant enzyme molecules, since we also get two different binding slopes indicating molecules with different binding properties to α-amanitin.

Furthermore we can say that there seems to be no increase of the resistant B enzyme in α-amanitin resistant cells, because we found the same number, namely 1×10^4 molecules of resistant polymerase per cell independent of growing the cells with or without the toxin.

It could be shown in control experiments that during preincubation of the enzymes for 12 h at 20°C before addition of [^{3}H] amanitin no enzyme proteolysis could be detected, since we found the same amount of binding with or without preincubation.

4. Discussion

Previous work from other laboratories has revealed that phenotypically stable mutants from different cell lines, resistant to the cytotoxic action of α-amanitin, possess an altered form of RNA polymerase B which is responsible for the drug resistant phenotype [1,2].

Somers et al. could show that the enzyme from α-amanitin resistant mutants of rat myoblast cells exhibit 30% resistance towards the toxin under in vitro conditions [4]. This suggests that this mutant cell line contains two wild type and one mutant allele of the genes determining the enzyme subunits involved in the binding of α-amanitin.

Based on the 50% resistant enzyme present in our MOPC 104 E Ama cell line at low amanitin concentrations it seems that this cell line is diploid for the amanitin binding subunit and that we have two alleles, one mutated and one wild type and that resistance is codominant.

In our MOPC 104 E cell line, however, a reduced level of sensitive enzyme could be observed when the

cells were grown in the presence of α-amanitin. The finding that less sensitive enzyme is present in cells grown on the toxin is due either to the fact that less sensitive enzyme is actually present under these conditions or that less sensitive enzyme is recovered during enzyme isolation in the presence of α-amanitin. This latter possibility could be due to a different behaviour of the α-amanitin bound enzyme during the isolation procedure. This question is under further investigation.

The amounts of resistant enzyme on the other hand, did not change appreciably whether the resistant cells were grown in α-amanitin or not, since we find in both cases about 1×10^4 resistant enzyme molecules per cell which is somewhat less than the numbers observed by Cochet-Meilhac et al. [8]. This observed constance of resistant enzyme differs from that of Somers et al. [5] who reported for different mammalian cell types an increase in the activity of resistant enzyme when resistant cells were transferred from α-amanitin-free medium to medium containing α-amanitin. At this point it is interesting to note that the MOPC 104 E ama cells grow more slowly in the presence than in the absence of the toxin. Thus in the absence of a regulatory mechanism the cells could be forced to grow with a suboptimal concentration of enzyme molecules to compensate for the functional loss of half of the RNA polymerase.

The fact that α-amanitin resistant cell lines from various sources yield different levels of wild type and mutant enzymes make these cell-lines interesting tools for studying the biosynthesis of RNA polymerase B and its regulation.

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