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Mammalian Cells with Altered Forms of RNA Polymerase II

(Chinese hamster ovary/α-amanitin)

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ABSTRACT **Mutants of Chinese hamster ovary cells that are resistant to α-amanitin can be isolated. At least some of these mutants contain an altered form of DNA-dependent RNA polymerase II, as indicated by its resistance to α-amanitin. These results indicate that mutation to α-amanitin resistance involves a change of a structural gene.**

Eukaryotes contain different forms of DNA-dependent RNA polymerase. Polymerase I is located in the nucleolus of the cell, whereas polymerase II, and perhaps a third form, polymerase III, are located in the nucleoplasm. Polymerases I and II can be differentiated by elution from DEAE-cellulose resins with salt; the sensitivity of polymerase II, and resistance of polymerase I, to the bicyclic peptide, α-amanitin; and the greater activity of polymerase with native versus denatured DNA as a template, whereas the reverse is true for polymerase II (1, 2). It is presumed that polymerases II and III are involved in the formation of heterogeneous mRNA (3). The presence of different forms of polymerase complicates the study of transcription in eukaryotic cells. Such studies could be facilitated if mutants involving one or all of the forms of the enzymes were available. RNA polymerase II is specifically inhibited by α-amanatin (4), which appears to interact with the enzyme to inhibit chain elongation (5–9). Mutants of cells resistant to α-amanitin might have altered forms of polymerase II. In this paper, we show that mutants of Chinese hamster ovary cells (CHO) resistant to α-amanitin can be isolated, and that at least one of these mutants contains an altered form of polymerase II.

MATERIALS AND METHODS

Cell Growth. CHO cells used in these experiments were a gift from Dr. W. C. Dewey and were carried routinely in exponential phase in suspension culture with α-medium (10) lacking nucleosides and supplemented with 10% dialyzed fetal-bovine serum. Cells were grown and plating efficiencies were determined by standard procedures (11).

Chemicals. α-Amanitin was a gift of Prof. T. Wieland, Max Planck Institute, Heidelberg, Germany.

Crude Extracts of Polymerase II were prepared essentially according to the method of Sugden and Sambrook (12). Basically the method involved the homogenization of 200–400 ml of about 10^8 log-phase cells in hypotonic Tris buffer (pH 7.9) and the subsequent recovery of the nuclei. Nuclei were treated with low-salt buffer to preferentially elute polymerase I. After elution, the solution was made 0.3 M in $(NH_4)_2SO_4$ and centrifuged at 45,000 rpm in a Spinco SW50.1 rotor for 1.5 hr at 4° to remove the particulate matter and chromatin. The supernatant was immediately assayed for DNA-dependent RNA polymerase activity with native calf-thymus DNA as template; the incorporation of [^{3}H]UMP into acid-insoluble material was measured.

Enzyme Extraction and Column Chromatography. DNA-dependent RNA polymerase enzymes were solubilized essentially according to the procedure of Sugden and Keller (manuscript in preparation). For each extraction, 2×10^9 log-phase cells were washed once with phosphate-buffered saline (pH 7.2) (13), and once with the homogenization buffer [10 mM Tris·HCl (pH 7.9)–0.1 mM EDTA–5 mM dithiothreitol]. At this stage, if desired, the cells were frozen at −70° for subsequent extraction. After washing, cells were swollen in a final volume of 25 ml of the homogenization buffer for 15–30 min, then disrupted by 3–5 strokes in a

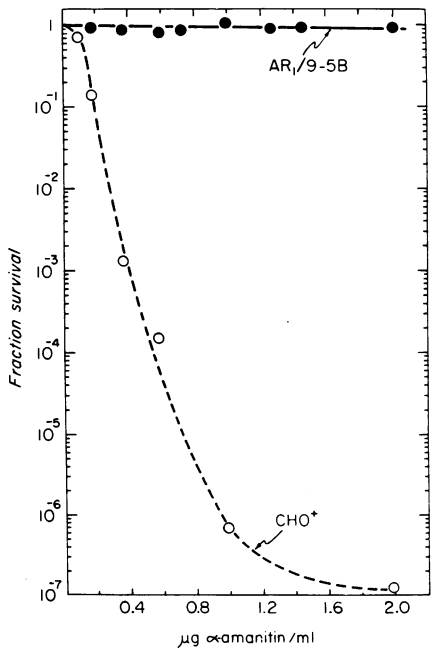

FIG. 1. Plating efficiency of wild type (CHO^+) (O– – –O) and an α-amanitin-resistant subline (AR_1/9-5B) (●——●), plated in the presence of α-amanitin at the concentrations shown.

Abbreviation: CHO, chinese hamster ovary.

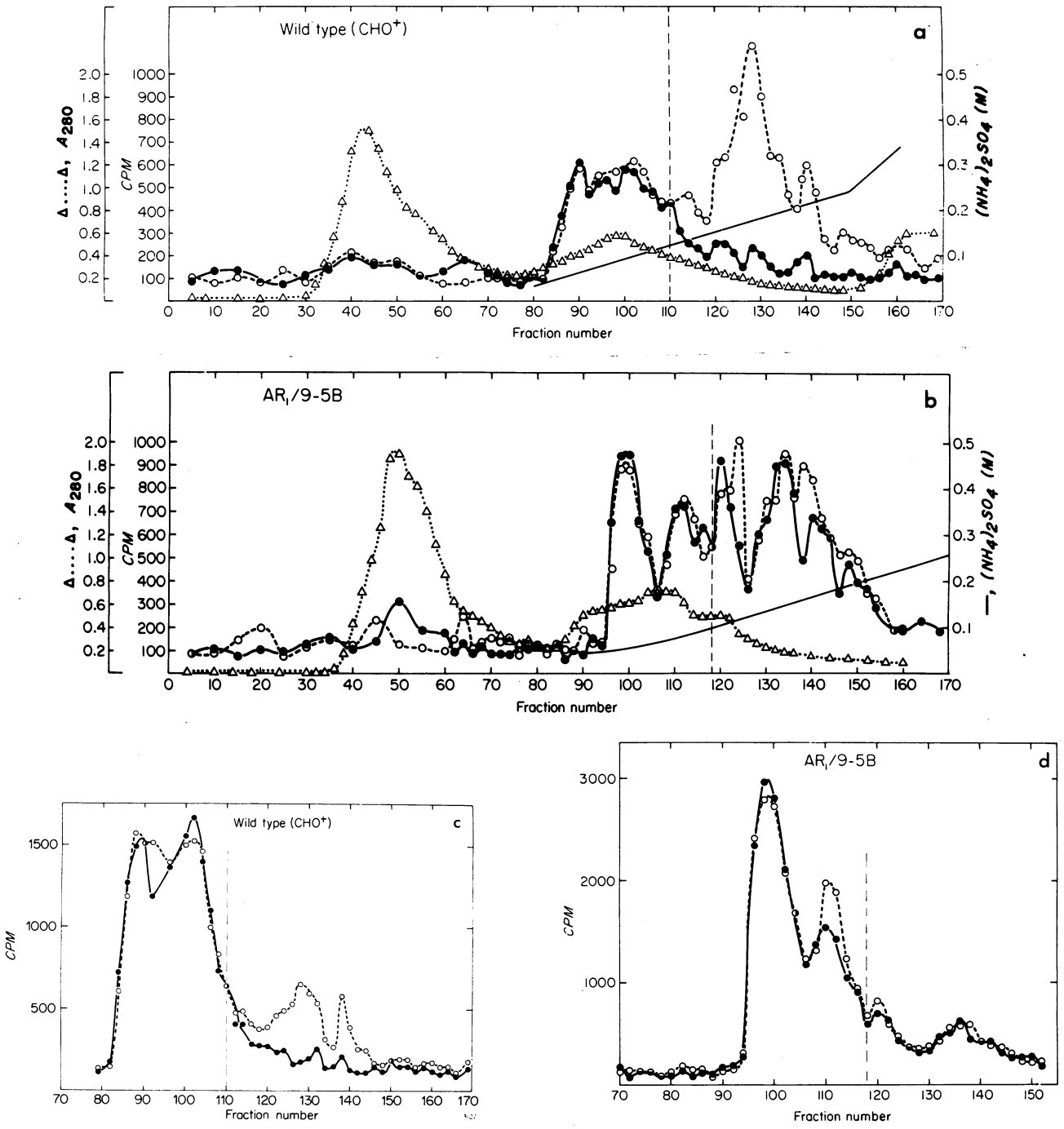

FIG. 2. DEAE-cellulose chromatography of soluble DNA-dependent RNA polymerase. Protein precipitated from the high-speed supernatant prepared from about 2×10^9 cells (see *Methods*) was suspended in 15 ml of standard buffer [50 M Tris·HCl (pH 7.9)–0.1 mM EDTA–0.5 mM dithiothreitol–25% glycerol–5 mM $MgCl_2$] containing 50 mM $(NH_4)_2SO_4$, dialyzed 8 hr against 300 ml of the same buffer, and centrifuged 1.5 hr at 45,000 rpm in a SW50.1 rotor to sediment the particulate matter. The supernatant was applied to a 1.8×25 cm DEAE-cellulose column equilibrated with 50 mM $(NH_4)_2SO_4$ in standard buffer. After washing twice with 10 ml (each time) of the standard buffer containing 50 mM $(NH_4)_2SO_4$, the enzyme was eluted with a linear gradient of $(NH_4)_2SO_4$ (50–500 mM) in the same buffer. 1.3-ml Fractions were collected and 25-μl aliquots were assayed as described in *Methods*. In Fig. 2*a* and *b*, the assay was with native calf-thymus DNA as a template, whereas in Fig. 2*c* and *d*, denatured calf-thymus DNA was used. ●—— ●, with 0.1 μg of α-amanitin per ml; O- - -O, without α-amanitin—, $(NH_4)_2SO_4$ molarity.

Dounce glass homogenizer. The disruption was monitored by phase-contrast microscopy and stopped when 80–90% of the cells had been converted to free nuclei. 2 ml of 0.5 M Tris·HCl (pH 7.9), 0.5 ml of 0.1 M dithiothreitol, 0.25 ml of 1 M $MgCl_2$, 0.025 ml of 0.2 M EDTA, 10 ml of glycerol, and 5 g of sucrose were added to the homogenate. The suspension was stirred until the sucrose was completely dissolved, H_2O was added to a final volume of 45 ml, and 5 ml of saturated $(NH_4)_2SO_4$ (pH 7.6) was then added with mild stirring. The extract was warmed to 35°, incubated for 45 min, cooled to 0° without stirring, and left for 4 hr. The particulate matter including DNA was removed by centrifugation in a

Spinco SW41 rotor at 35,000 rpm for 3.5 hr. The protein was precipitated by the addition of 0.34 g of $(NH_4)_2SO_4$ per ml. The precipitate was collected by centrifugation in a SW41 rotor at 35,000 rpm for 1 hr, suspended in 15 ml of standard buffer (pH 7.9) containing 0.05 M $(NH_4)_2SO_4$, and treated as described in the legend of Fig. 2.

DNA-Dependent RNA Polymerase was routinely assayed in 100 μl containing: 0.5 mM each of ATP, CTP, and GTP (Calbiochem); 25 μg of calf-thymus DNA (Sigma) per ml; 0.1 mM dithiothreitol; 1.5 mM $MnCl_2$; 75 mM Tris·HCl (pH 7.9); 0.125 mM EDTA; 25 mM $(NH_4)_2SO_4$; 0.5 mg of crystallized bovine-serum albumin per ml; 0.1 mM [^{3}H]UTP (0.1 Ci/mol); and enzyme. The mixture was incubated at 35° for 25 min, and the reaction was terminated by the addition of 0.1 ml of bovine-serum albumin (25 mg/ml) followed immediately by 3 ml of ice-cold 5% Cl_3CCOOH–0.01 M $Na_4P_2O_7$. The precipitate was collected on glass-fiber filters (Whatman GF/C, 24 mm), washed six times with cold 5% Cl_3CCOOH, once with ethanol, dried, and counted in a toluene-based scintillation fluid (14).

RESULTS AND DISCUSSION

Isolation of cells resistant to α-amanitin

In an attempt to define the appropriate conditions for the isolation of CHO cells that are resistant to the lethal effects of α-amanitin, preliminary growth and plating experiments were performed. As will be shown later (Fig. 3) the concentrations of α-amanitin required for 50% and essentially complete inhibition of RNA polymerase II activity, after partial purification on DEAE-cellulose columns, are 0.012 and 0.05 μg/ml, respectively. However, when cells were exposed to 1.0 μg of α-amanitin per ml for various lengths of time, then plated in the absence of the drug, an 18-hr exposure to the drug reduced the plating efficiency by only 50%. Furthermore, exposure of mass populations of cells to the same drug concentration and determinations of cell growth indicated that there was an approximate tripling of the cell number, indicating that the lethal action of the drug is not rapid. When CHO cells were plated in the presence of 0.1 μg of α-amanitin per ml, essentially no effect on plating efficiency was seen. However, when cells were plated in medium with 1.0 μg of α-amanitin per ml the plating efficiency was reduced to about 7×10^{-7}. It appears that either the effective concentration of α-amanitin within the cell is much less than that in the surrounding medium or that a loss of the products of RNA polymerase II activity is not immediately lethal to the cells.

It appeared that an effective method of selection of resistant cells would be to plate the cells in the continuous presence of 0.1 μg of drug per ml. Isolation was performed with an untreated population and with populations mutagenized either once or twice. In the experiments involving the mutagen, cells were exposed to 200 μg of ethyl methane sulfonate per ml for a period of 16 hr at 34°, and in the case of the double treatment with mutagen, the above procedure was repeated after an interval of 1 week. The survival after a single treatment with mutagen was 64%, and the interval between final mutagenesis and plating in α-amanitin was 3–4 days. In all three cases, the colonies that grew in the presence of 1.0 μg of α-amanitin per ml were individually picked and grown into mass populations again in the presence of the same drug concentration. Cells were then cloned again and grown into mass populations in the absence of the drug. Each subline so isolated was tested for sensitivity to the drug. The plating efficiencies during the first

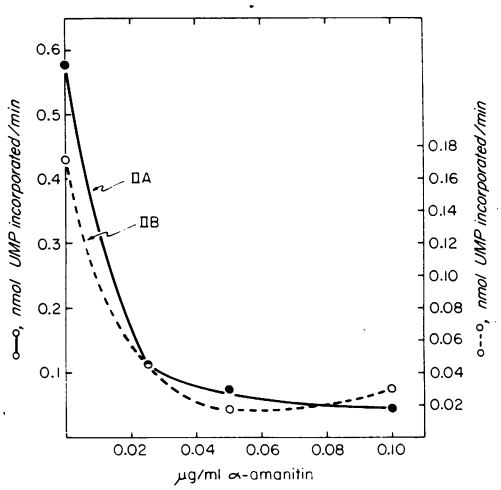

FIG. 3. Effect of α-amanitin on wild-type, DNA-dependent RNA polymerase. For these studies, RNA polymerase eluted from the column described in Fig. 2 was used. Assay conditions were identical to those described in *Methods;* denatured calf-thymus DNA was used as a template. The *solid line* represents data from enzymes prepared from fraction 128 in Fig. 2*a* or *c*, whereas the *dashed line* represents data from fraction 140 in Fig. 2*a* or *c*.

TABLE 1. *Growth sensitivity and RNA polymerase activity in cells resistant to α-amanitin*

Subline	Growth sensitivity to α-amanitin*	Percent inhibition of DNA-dependent RNA polymerase by α-amanitin†
CHO$^+$	*S*	50
AR_1/9-5B	*R*	0.8
AR_1/10-1a	*R*	2.7
AR_1/13-1	*R*	0.7
AR_1/25-2A	*R*	6.3
AR_2/21-2A	*R*	46
AR_1/8-1	*R*	38
AR_1/5-2A	*R*	15
AR_1/45-2A	*R*	21.8

RNA polymerase activities in the high-speed supernatant (ref 12 and text) were prepared from 1 to 2 × 10^8 log-phase cells. The protein concentration of the supernatant ranged from 0.08 to 0.25 mg/ml; 50 μl was used for each reaction mixture. The conditions of the assay were as described in *Methods*, except that the total volume of the reaction mixture was 200 μl, the $(NH_4)_2SO_4$ concentration was 50 mM, and the template was always native calf-thymus DNA. The concentration of α-amanitin used was 25 ng/ml. The values given for the first five strains are means of three determinations from three independent experiments, whereas the other values are based on a single determination. In every case, for each determination, duplicate samples were run in the presence or absence of α-amanitin. CHO, Chinese hamster ovary.

* *R* indicates that the plating efficiency of the subline was independent of the presence of α-amanitin up to a concentration of 1.0 μg/ml. *S* indicates the sensitivity illustrated in Fig. 1.

† Enzyme was assayed in cell-free extracts.

isolation step were less than 10^{-6}, 1.8×10^{-5}, and 3.0×10^{-5} for the untreated, once-mutagenized, and twice-mutagenized populations, respectively. At the end of the second cloning, more than 100 clones from each mutagenized population were tested for resistance to α-amanitin, and all were resistant. Fig. 1 shows curves of plating efficiency against drug concentration, in the plating medium, for the wild-type subline (CHO^+) and for one of the resistant sublines, $AR_1/9$-5B. Other resistant sublines behaved similarly. Column 2 of Table 1 is a summary of the plating efficiency data for CHO^+ and eight resistant sublines. The symbol *R* is used to indicate that up to a concentration of 1.0 μg of α-amanitin per ml, the plating efficiency of the subline was essentially independent of the drug. Repeated tests of the stability of the resistant phenotype with several of these sublines grown in medium lacking α-amanitin for periods of up to 6 months indicated no measurable loss of resistance to α-amanitin.

Assay of RNA polymerase activity in cell-free preparations

To test whether α-amanitin-resistant cells did in fact have an altered RNA polymerase, enzymatic activity was assayed in crude cell-free extracts. The average inhibition of RNA polymerase activity from the CHO^+ cells was 50%, with a range from 30 to 70%; this is to be expected because of residual contamination with RNA polymerase I resistant to α-amanitin. Column 3 of Table 1 indicates that among eight sublines resistant to α-amanitin, four appeared to contain an enzyme with markedly increased resistance to α-amanitin, two showed an enzyme with a moderate degree of resistance to the drug, and the remaining two exhibited a response that was very similar to that of the CHO^+ cell line.

In an attempt to further increase the sensitivity of the polymerase assays and to determine whether the resistance to α-amanitin was actually due to an altered form of RNA polymerase II, the crude extracts of the wild-type CHO cells and one of the mutants, $AR_1/9$-5B, were further purified by chromatography on DEAE-cellulose columns with a linear gradient of $(NH_4)_2SO_4$ for elution. Fractions were assayed for their ability to convert [^{3}H]UTP into acid-insoluble material with either native DNA (Fig. 2*a* and *b*) or denatured DNA (Fig. 2*c* and *d*), as templates, in the presence or absence of α-amanitin or rifampycin.

Fig. 2*a* and *c* show that RNA polymerase activity in the wild-type cells was localized within four major peaks. The first two peaks (to the left of the dashed line) are presumably two forms of RNA polymerase I, since they are eluted from the column by relatively lower salt concentrations than those required for polymerase II, they are completely resistant to 0.1 μg of α-amanitin per ml, and their activities are higher with native DNA than with denatured DNA (compare Fig. 2*a* and *c*). It was possible that one or both of these peaks consisted of mitochondrial RNA polymerase, since this enzyme derived from *Neurospora* (15), rat liver (16), and fungus B. *emersoni* (17) is also resistant to α-amanitin. However, this possibility was ruled out by the finding that both forms of enzyme from CHO cells, tentatively identified as polymerase I, were resistant to 6 μg of rifampycin per ml (data not included), whereas the mitochondrial enzyme is sensitive to this concentration of rifampycin (15–17).

The third and fourth peaks of activity isolated from wild-type cells (to the right of the dashed line), are presumably different forms of RNA polymerase II, since they are eluted at a higher salt concentration than polymerase I, they are both sensitive to α-amanitin (Figs. 2*a* and *c*, and 3), and denatured DNA is the more effective template for expression of their activity (Fig. 2*a* and *c*). Two forms of RNA polymerase II have been observed by other authors (18–20).

These data support the contention of others that both RNA polymerase I and II may exist in several forms, which are at least partially separable on DEAE-cellulose columns. These forms are presumably due to different combinations of subunits as proposed by Kedinger *et al.* (19). Different forms of RNA polymerase I and II have only been isolated in a few cell types; thus, it is possible that the presence of the various forms is a reflection on the state of differentiation of the particular cell, although the possibility that the different forms were due to alterations (e.g., proteolytic degradation) induced in the enzyme during extraction is not excluded.

Four major peaks of RNA polymerase activity were also found in extracts from the subline resistant to α-amanitin, $AR_1/9$-5B (Fig. 2*b* and *d*). The first two peaks were similar to those isolated from the wild-type cell in that they were eluted from the column at the same ionic strength, showed complete resistance to α-amanitin, and native DNA was the more effective template for expression of their activity. However, the behavior of the RNA polymerase activity to the right of the dashed line differed from that of the wild type in several respects. First, the enzyme activity from the mutant was completely resistant to 0.1 μg of α-amanitin per ml, which essentially abolished the enzyme activity from the corresponding region in the wild-type cell (Fig. 3). Secondly, the profile of the peaks also seemed to be altered in the mutant strain. Thus, it would appear that the CHO mutant, $AR_1/9$-5B, possesses a mutation in the RNA polymerase II cistron, *Aman* (amanitin), which produces the protein that interacts with α-amanitin. Presumably, the other mutants in Table 1 that exhibit similar resistant enzyme properties, also possess an *Aman* mutation, although the properties of the partially purified enzymes have not been tested.

In summary, we have shown that mutants of CHO cells that are resistant to α-amanitin can be isolated and that at least some of these mutants contain an altered RNA polymerase II. Whether the other sublines that exhibit marked resistance to the killing effects of the drug, but show a less pronounced resistance to inhibition of the enzyme by α-amanitin, reflect lesser changes in the enzyme, or other mechanisms leading to cell resistance, remains to be determined.

These studies are of interest in respect to the nature of mutations in somatic cells. Recently, Harris (21) and Mezger-Freed (22) have raised serious questions concerning the chromosomal nature of inherited changes in such cells (23). In the absence of techniques that allow recombination and segregation of genetic markers, it is indeed difficult to ascertain whether or not an inherited modification is Mendelian in nature. Nevertheless, as shown here, mutations to α-amanitin resistance do resemble structural gene changes. The genetic change in this case seems to involve a physical modification in the RNA polymerase enzyme, and the simplest hypothesis to explain this result is that we are dealing with a change in a structural gene. Confirmation of this hypothesis requires further characterization of the genetic change and the nature of the modification in the enzyme.

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