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Abstract

A permeable cell system for studying RNA synthesis was established. Mouse ascites sarcoma cells were made permeable to nucleoside triphosphates and alpha-amanitin by treating with a hypotonic buffer. Separate determinations of endogenous RNA polymerase I, II and III activities in permeable cells were conducted using the different sensitivities of these enzymes to alpha-amanitin. The endogenous activity of RNA polymerase II under optimal conditions was one tenth of total RNA synthetic activity in isolated nuclei, and one third of that in permeable cells. The extremely low ratio of RNA polymerase II activity to total RNA synthetic activity in isolated nuclei was thought to be caused by increase of RNA polymerase I activity and decrease of RNA polymerase II activity. These and other results suggested that RNA synthesis in permeable cells reflects more precisely the in vivo state of RNA synthesis than thatin isolated nuclei. The permeable cell system will provide a useful method for studying the separate activities of RNA polymerases I, II and III in situ.

KEYWORDS: RNA synthesis, RNA polymerases I, II and III, mouse ascites sarcoma cells, permeable cewlls, ?-amanitin

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RNA SYNTHESIS IN PERMEABLE MOUSE ASCITES SARCOMA CELLS

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Abstract. A permeable cell system for studying RNA synthesis was established. Mouse ascites sarcoma cells were made permeable to nucleoside triphosphates and α-amanitin by treating with a hypotonic buffer. Separate determinations of endogenous RNA polymerase I, II and III activities in permeable cells were conducted using the different sensitivities of these enzymes to α-amanitin. The endogenous activity of RNA polymerase II under optimal conditions was one tenth of total RNA synthetic activity in isolated nuclei, and one third of that in permeable cells. The extremely low ratio of RNA polymerase II activity to total RNA synthetic activity in isolated nuclei was thought to be caused by increase of RNA polymerase I activity and decrease of RNA polymerase II activity. These and other results suggested that RNA synthesis in permeable cells reflects more precisely the *in vivo* state of RNA synthesis than thatin isolated nuclei. The permeable cell system will provide a useful method for studying the separate activities of RNA polymerases I, II and III *in situ*.

Key words: RNA synthesis, RNA polymerases I, II, and III, mouse ascites sarcoma cells, permeable cells, α-amanitin.

The mechanism of genetic transcription in eukaryotic cells has been studied *in vivo* in intact cells and *in vitro* by either purified enzyme-template system or isolated nuclei (1–3). RNAs synthesized *in vivo* are classified into three classes: ribosomal RNA (4, 5), heterogeneous nuclear RNA (4, 6) and small molecular weight nuclear RNA (7). These are synthesized by RNA polymerases I, II and III, respectively. Endogenous RNA polymerases I, II and III differ from each other by their *in vitro* sensitivity to α-amanitin (7, 8). *In vivo* states of ribosomal RNA, heterogenous nuclear RNA and small molecular RNA synthetic activities have been studied *in vitro* by developing a proper *in vitro* system for assaying endogenous RNA polymerase I, II and III activities (2, 9). Purified RNA polymerase activities do not necessarily reflect *in vivo* activities (10). In a nuclear system, RNA polymerase I activity is highly dependent on magnesium ion at low ionic strength, whereas RNA polymerase II activity is highly dependent on manganese ion (1–3). Such a difference in ionic conditions is not expected *in vivo*, where RNA polymerases I and II synthesize RNA, simultaneously (9). Nuclei

isolated in a sucrose medium lack the capacity of synthesizing DNA-like RNA which is synthesized by RNA polymerase II (3). These findings indicate that there are unclarified gaps in RNA synthesis between nuclei and intact cells. In the present communication, permeable cells were studied to obtain a preparation with intermediate properties between intact cells and nuclei.

MATERIALS AND METHODS

Mouse ascites sarcoma cells (SR-C3H/He cells) induced in a C3H/He mouse by the Schmidt-Ruppin strain of Rous sarcoma virus by Yamamoto and Takeuchi (11) were injected intraperitoneally into C3H/He mice. Ascites cells were collected from the ascites fluid of mice 7 days after ascites inoculation of 0.05 ml/mouse (0.5 to 1.0×10^7 cells). [^{3}H]-UTP was purchased from the Radiochemical Center, Ltd., England, and α-amanitin and unlabelled nucleoside triphosphates were obtained from Boehringer Manheim, Germany.

Preparation of permeable cells. Ascites fluid was chilled in an ice bath and centrifuged at $170\times g$ for 5 min. Precipitated cells were washed three times by centrifugation in a solution containing 10 mM Tris-HCl, pH 7.4, 0.14 M NaCl, 1 mM Na_2HPO_4, and 5 mM KCl. The cells were suspended at 5×10^6 cells/ml in a hypotonic buffer (TCMB buffer) containing 10 mM Tris-HCl, pH 7.9, 1 mM $CaCl_2$, 0.4 mM $MgCl_2$ and 6 mM 2-mercaptoethanol and stood at 4°C for 15 min. One tenth volume of 2.6 M sucrose containing 10 mM Tris-HCl, pH 7.4 at 4°C, 6 mM $MgCl_2$ and 6 mM 2-mercaptoethanol were added to this suspension with gentle stirring. After precipitation the cells were suspended in 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4 at 4°C, 6 mM $MgCl_2$, and 6 mM 2-mercaptoethanol (buffer A).

Isolation of nuclei. Permeable cells (10^7 cells/ml) were homogenized in buffer A with a Dounce homogenizer (Kontes Glass Corp., Vineland, N. J.) and centrifuged at $170\times g$ for 5 min. The cells were suspended in buffer A, then 4.8 volumes of 2.6 M sucrose solution was added with stirring. This suspension was centrifuged at $45{,}000\times g$ for 60 min. The precipitated nuclear fraction was suspended in buffer A.

Assay of RNA polymerase activity. The standard reaction mixture contained 50 mM Tris-HCl, pH 7.9 at 25°C, 6 mM $MgCl_2$, 0.4 mM each of ATP, GTP and CTP, 0.04 mM unlabelled UTP, 1 μCi of [^{3}H]-UTP (10–20 Ci/mmol), 1 mM dithiothreitol, and 100 mM KCl in a volum of 0.1 ml. Variation in the constituents of the standard reaction medium are indicated in the legends for figures. The reaction mixture was incubated at 25°C for 10 min, then the reaction was stopped by chilling the tubes in an ice water bath. Two ml of cold 5% trichloroacetic acid containing 0.02 M sodium pyrophosphate was then added to each tube. The acid-precipitable fractions were collected on glass fiber filters and washed with ice-cold 5% trichloroacetic acid solution. The filters were dried and the radioactivity was counted.

RNA polymerases I, II, and III were identified by their sensitivity to α-amanitin. RNA polymerase II activity is completely inhibited at 0.5 μg/ml of

α-amanitin (7), RNA polymerase III is inhibited at higher α-amanitin concentration (8) (50% inhibition at 10 to 20 μg/ml), and RNA polymerase I shows no sensitivity to α-amanitin (7). RNA polymerase I activity was assayed in the presence of 100 μg/ml of α-amanitin, and RNA polymerase II activity was estimated by substraction of the activity in the presence of 0.5 μg/ml α-amanitin from the activity without α-amanitin. RNA polymerase III activity was determined by subtraction of the activity in the presence of 100 μg/ml α-amanitin from the activity in the presence of 0.5 μg/ml α-amanitin.

DNA was determined according to the procedure of Burton (12).

RESULTS

Suspension of SR-C3H/He cells in the hypotonic (TCMB) buffer for 10 min rendered the cells permeable to nucleoside triphosphates (Fig. 1). The time course of RNA synthesis in permeable cells at 25°C, 30°C and 37°C is shown in

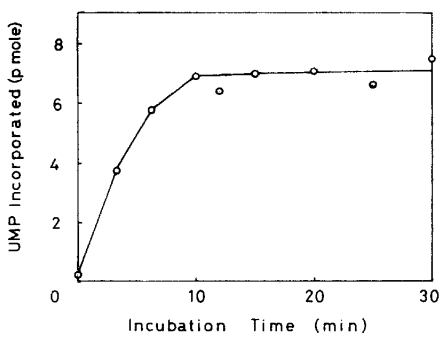

Fig. 1. Effect of hypotonic treatment on the ability of SR-C3H/He cells to incorporate [^{3}H]UTP into RNA. SR-C3H/He cells were suspended in hypotonic buffer (TCMB buffer) at a concentration of 5×10^6 cells/ml. After incubation at the indicated time, the cells were centrifuged and suspended in buffer A. RNA synthesis was assayed under standard conditions described in Materials and Methods.

Fig. 2A. At 37°C polymerase activity was higher than at 25°C. At lower temperatures, however, degradation of synthesized RNA was minimal for prolonged incubation periods. An increased extent and duration of RNA synthesis were observed in the presence of a high concentration of nucleoside triphosphates (Fig. 2B). At 0.12 mM of UTP the time course of RNA synthesis at 25°C in permeable cells was linear for 15 min, then proceeded for 60 min at a diminished rate.

Permeable cells have been shown to be permeable to histone and other polypeptides (13). α-amanitin is an octapeptide and is not freely permeable to living cells. Hypotonic treatment rendered cells permeable to α-amanitin. RNA synthesis in permeable cells was inhibited stepwise by α-amanitin (Fig. 3). On the

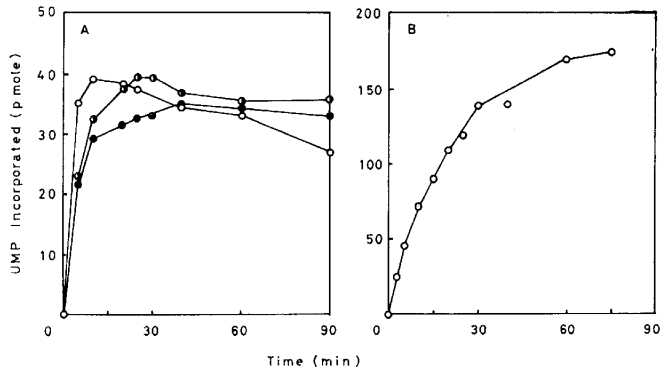

Fig. 2. Time course of RNA synthesis in permeable cells. A). RNA synthesis in permeable cells (35 μg DNA) was assayed under standard conditions at the indicated temperature, and for the indicated time. ○——○, 37°C; ◑——◑, 30°C; ●——●, 25°C. (B) RNA synthesis in permeable cells assayed at 0.12 mM UTP at 25°C.

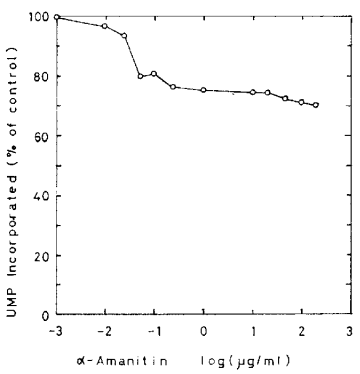

Fig. 3. Effect of α-amanitin on RNA synthesis in permeable cells. Permeable SR-C3H/He cells (74 μg DNA) were assayed under standard conditions in the presence of the indicated amounts of α-amanitin.

basis of difference in sensitivity to α-amanitin (7, 8), the first step of inhibition in Fig. 3 resulted from inhibition of RNA polymerase II and the second step of inhibition resulted from inhibition of RNA polymerase III.

Optimal KCl and $MgCl_2$ concentrations for RNA synthesis in permeable cells were similar to those in isolated nuclei. The effect of KCl concentration on RNA synthesis was biphasic and optimal concentrations were 0.1 M and 0.4 to 0.6 M (Fig. 4A, 5A). At KCl concentrations between 0.4 and 0.6 M, RNA synthesis in the presence of Mn^{++} was higher than RNA synthesis in the presence of Mg^{++}. Optimal $MgCl_2$ concentration for RNA polymerases I, II and III in permeable cells was 4 to 8 mM (Fig. 4B). RNA polymerases I, II and III in

isolated nuclei have activity in a broad range of Mg^{++} concentration (2 to 12 mM) (Fig, 5B). Mn^{++} requirement varied with the polymerase (Fig. 4C, 5C). The optimal Mn^{++} concentration for polymerase I activity in permeable cells was about 1 mM. Polymerase I activity in isolated nuclei was inhibited by Mn^{++} over 2 mM. The optimal Mn^{++} concentration for RNA polymerase II activity in permeable cells and isolated nuclei was between 1 and 3 mM, and for RNA polymerase III, between 0.5 and 2 mM.

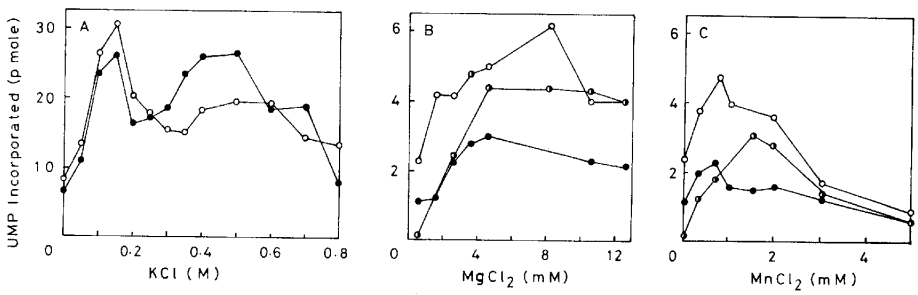

Fig. 4. Effects of metal ion concentrations on RNA synthesis in permeable SR-C3H/He cells. (A) Effects of KCl concentrations on RNA synthesis in permeable cells (37 μg DNA). ○——○, in the presence of 6 mM $MgCl_2$; ●——●, in the presence of 1 mM $MnCl_2$. (B) Effects of $MgCl_2$ concentrations on RNA synthesis in permeable cells (16 μg DNA). ○——○, RNA polymerase I activity; ◑——◑, RNA polymerase II activity; ●——●, RNA polymerase III activity. (C) Effects of $MnCl_2$ concentrations on RNA synthesis in permeable cells (16 μg DNA). ○——○, RNA polymerase I activity; ◑——◑, RNA polymerase II activity; ●——●, RNA polymerase III activity.

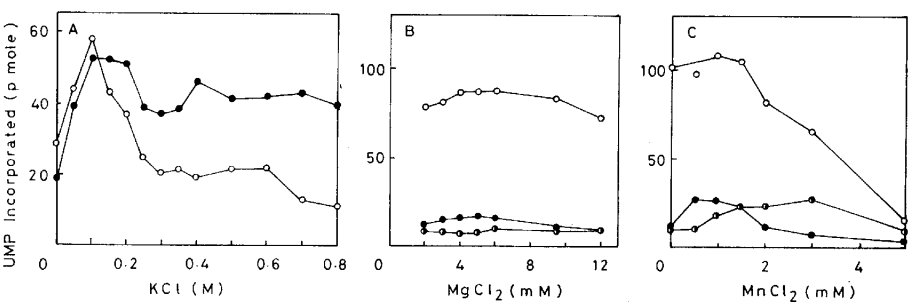

Fig. 5. Effects of metal ion concentrations on RNA synthesis in isolated nuclei. (A) Effect of KCl concentrations on RNA synthesis in isolated nuclei (172 μg DNA). ○——○, in the presence of 6 mM $MgCl_2$; ●——●, in the presence of 1 mM $MnCl_2$. (B) Effect of $MgCl_2$ concentrations on RNA synthesis in isolated nuclei (132 μg DNA). ○——○, RNA polymerase I activity; ◑——◑, RNA polymerase II activity; ●——●, RNA polymerase III activity. (C) Effect of $MnCl_2$ concentrations on RNA synthesis in isolated nuclei (132 μg DNA). ○——○, RNA polymerase I activity; ◑——◑, RNA polymerase II activity; ●——●, RNA polymerase III activity.

Figs. 4B and 5B show that endogenous RNA polymerase II activity in the presence of optimal $MgCl_2$ and 0.1 M KCl in isolated nuclei was about one tenth of the total nucler RNA synthesis activity. In permeable cells, endogenous RNA polymerase II activity under the same conditions was about one third of the total RNA synthesis activity.

RNA polymerase activity was studied in permeable cells under standard conditions and in isolated nuclei. RNA polymerase I activity in permeable cells (Fig, 6A) was one half that of isolated nuclei (Fig. 6B). RNA polymerase II activity in the presence of Mg^{++} in permeable cells (Fig. 6A) was 1.4 times that of

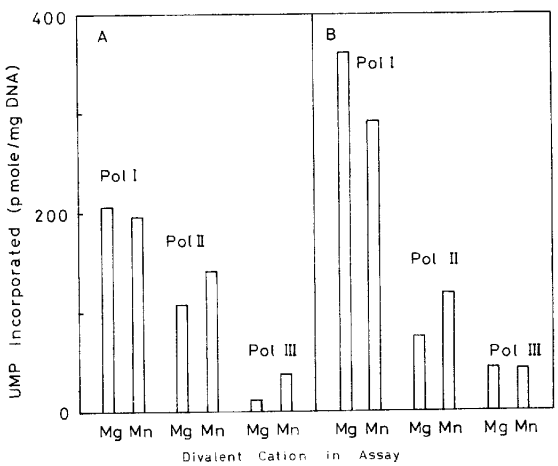

Fig. 6. Comparison of RNA polymerase activity in permeable cells and isolated nuclei. RNA polymerase I, II and III activities were assayed in the presence of 6 mM $MgCl_2$ and 0.1 M KCl or in the presence of 1 mM $MnCl_2$ and 0.1 M KCl as indicated. RNA polymerase activities were standardized for 1 mg DNA of permeable cells or nuclei. (A), RNA polymerase activity in permeable cells. (B), RNA polymerase activity in isolated nuclei.

isolated nuclei (Fig. 6B). Decrease in the ratio of RNA polymerase II activity to total RNA synthetic activity in isolated nuclei caused by increase of RNA polymerase I activity and decrease of RNA polymerase II activity in isolated nuclei.

DISCUSSION

Isolated nuclei provide a system for studying transcription (1-3), maturation (14) and transport (15) of RNA from the nucleus. In isolated nuclei, endogenous RNA polymerase I activity is dependent on Mg^{++} and endogenous RNA polymerase II activity is dependent on both Mn^{++} and high concentration of monovalent cations (1-3). Autoradiographic studies have shown that nucleoplasmic and nucleolar RNA synthesis occur simultaneously *in vivo* (9). There are un-

clarified gaps between *in vivo* and *in vitro* nuclear systems. The nucleotide permeable cells were also permeable to α-amanitin, therefore it was possible to determine endogenous RNA polymerase I, II and III activities. The present experiments showed that, in permeable cell systems, RNA polymerases I and II both synthesized RNA in the presence of Mg^{++} and 0.10 to 0.15 M KCl. Mn^{++} has been shown to be mutagenic *in vivo*, as well as reducing the fidelity of DNA synthesis *in vitro* (16), and the fidelity of transcription of ribosomal RNA (17). These resuls suggest that RNA synthesis assayed in the presence of Mg^{++} rather than Mn^{++} reflects RNA synthesis in intact cells. Since divalent cation requirements of RNA polymerase II vary with template structure (18), the fact that RNA polymerase II activity in the presence of Mg^{++} is lower in isolated nuclei than in permeabl cells suggests that isolation of nuclei may involve alteration of the template structure. So far, permeable cell systems have not been adequately studied for endogenous RNA synthesis. The present paper shows that permeable cell systems are a useful method for studying endogenous RNA synthesis.

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