Inhibition of Transcription by Mammalian Ribonucleic Acid Polymerase II: Effects of Diethylstilbestrol and Its Analogues

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We examined the effects of diethylstilbestrol (1), *Z,Z*-dienestrol (2), *E,E*-dienestrol (3), indenestrol A (4), indenestrol B (5) and estradiol (6) on the activity of *in vitro* accurate transcription from adenovirus 2 major late promoter by mammalian ribonucleic acid (RNA) polymerase II using partially purified transcription factors from HeLa cell nuclear extract as well as on RNA polymerase II activity assayed for random incorporation of ribonucleotides. 1, 2, and 3 inhibited both activities, whereas 4 and 5 were inhibitory only in the transcription activity at concentrations higher than 10 $\mu\text{g/ml}$. However 6 had no effects on both activities.

Keywords transcription; RNA polymerase II; diethylstilbestrol; indenestrol A; indenestrol B; estradiol; dienestrol

trans-Diethylstilbestrol (1) is a potent synthetic estrogen capable of eliciting biological responses similar to those induced by endogenous 17 β -estradiol.¹⁾ In recent years, studies²⁾ in humans and mice have suggested that *in utero* exposure to 1 induces cancerous lesions of the reproductive tract. The mechanism of these effects of 1 is unknown, in spite of many studies covering metabolic activation,³⁾ deoxyribonucleic acid (DNA) binding,⁴⁾ radical formation⁵⁾ or DNA breaks.⁶⁾ We have reported that 1 and its derivatives inhibit microtubule assembly *in vitro*.⁷⁾ Also, ²H-labeled 1 has been analyzed by means of deuterium nuclear magnetic resonance (NMR), which demonstrated its intercalation into multibilayers of egg phosphatidylcholine.⁸⁾ Moreover, we have elucidated that 1 and its derivatives caused aneuploidy induction and disturbance of microtubule distribution in Chinese hamster V79 cells.⁹⁾ These findings suggest that within cells, 1 does not act directly on DNA.

Accurate transcription initiation by mammalian ribonucleic acid (RNA) polymerase II *in vitro* is recognized as a complex reaction involving multiple factors, and is a major regulatory step in many biological phenomena.^{10–12)} Several laboratories, including ours, have identified proteins which are required for correct transcription initiation. Some of these factors have been partially purified and characterized,^{13–19)} and can be separated functionally into two or more groups.

We are interested in the direct effect of diethylstilbestrol (1) on the transcription factors and mammalian RNA polymerase II in eukaryotic transcription. Therefore, in the present study, we examined the effect of 1, *Z,Z*-dienestrol (2) and indenestrol A (4),²⁰⁾ the latter two being the products of *in vivo* and *in vitro* oxidative metabolism³⁾ of 1, *E,E*-dienestrol (3) and indenestrol B (5), which are chemical analogs of 3 and 4, respectively, and estradiol (6), which is the most potent naturally occurring estrogen in mammals for *in vitro* transcription by mammalian RNA polymerase II using the partially purified transcription factors and mammalian RNA polymerase II.

Experimental

Materials Diethylstilbestrol (1), *E,E*-dienestrol (3), and estradiol (6) were purchased from Sigma Chemical Co. Syntheses of *Z,Z*-dienestrol (2) and indenestrols A and B (4 and 5) were performed as described elsewhere.²⁰⁾ Chart 1 shows the chemical structures of the six drugs used in this study. Hind III was purchased from Takara Shuzo Co., Ltd. [α -³²P]uridine triphosphate (UTP) was from Du Pont-New England

Nuclear. Other materials were of reagent grade.

Plasmids and Template The template used for transcription was a plasmid, pAd2 Δ , which contained the adenovirus type 2 major late (ML) promoter sequence. The length of the transcript resulting from run-off assay was 186 nucleotides when this template was digested with Hind III. The template used for RNA polymerase activity was denatured salmon sperm DNA.

Preparation of Transcription Factors HeLa cell nuclear extract (7.5 g) was prepared as described,²¹⁾ and transcription factors, FA, FB, FC, FD, FE, and FF were resolved by chromatographic procedures as reported previously.¹⁹⁾

***In Vitro* Transcription Activity** *In vitro* transcription assay was carried out in a 27.5- μl reaction volume containing 11 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 55 mM NaCl, 10% glycerol, 500 μM each of citidine triphosphate (CTP), guanosine triphosphate (GTP) and adenosine triphosphate (ATP), 25 μM [α -³²P]UTP (approximately 2 μCi), 0.5 μg of pAd2 Δ and transcription factors as indicated in Figures. The addition of drug/acetone was usually 1 μl . The reaction was started by the addition of the nucleoside triphosphate mixture. After incubation at 29 °C for 60 min, the reaction was stopped by adding 100 μl of 20 mM Tris-HCl pH 7.9, 1 mM EDTA, 0.3 M NaCl, 7 M urea, and 0.5% sodium dodecyl sulfate (SDS). Transcripts were purified and analyzed by electrophoresis on 6% polyacrylamide–7 M urea gel. The gels were then exposed to Fuji X-ray film at –80 °C with an intensifying screen. The inhibition activities were determined by densitometric analysis of the exposed X-ray film using a Bio-rad model 620 video densitometer.

Preparation of RNA Polymerase II RNA polymerase II was partially purified from calf thymus as described by Hodo and Blatti²¹⁾ except that the final step of purification on a column of agarose A-1.5 m capped with Sephadex A-25 was omitted. The purity of RNA polymerase II was estimated to be 94% among RNA polymerases by α -amanitin inhibition assay.

Assay of RNA Polymerase Activity The assays were done in a final volume of 27.5- μl containing 10 mM Tris-HCl (pH 7.9), 100 mM NaCl, 1.8 mM MnCl₂, 180 μM each of GTP, CTP and ATP, 9 μM [α -³²P] UTP (approximately 2 μCi), and 8 μg of DNA. The reaction was initiated by the addition of 5 μl of enzyme and incubated at 37 °C for 30 min; 30 μl aliquots were withdrawn from each tube after the addition of 2.5 μl of 50 mM UTP, and spotted onto DEAE-cellulose paper filters. The filters were washed six times for 5 min each in 5% Na₂HPO₄, then in distilled water and in 95% EtOH, and finally dried under an infrared lamp. Radioactivity was determined by placing each disc in 10 ml of aqueous counting scintillant (ACS II: Amersham Corporation).

Other Procedures SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli,²²⁾ and gels were stained with Ag-stain obtained from Daiichi. Protein concentration was measured by the dye-binding method²³⁾ or absorbance at 280 nm using bovine serum albumin as a standard.

Results and Discussion

First, we examined the effect of diethylstilbestrol (1) on *in vitro* accurate transcription from adenovirus 2 major late promoter by mammalian RNA polymerase II using partially

Fig. 1. Effect of Diethylstilbestrol (**1**) on Transcription by RNA Polymerase II

In vitro transcription of pAd2A was assayed as described in Experimental by using FA (3.0 μl), FB (1.5 μl), FC (3.0 μl), FD (3.0 μl), FE (4.0 μl), FF (1.5 μl) and purified RNA polymerase II (1.0 μl). Arrow indicates correct transcript. A) An autoradiogram from the results of the assay: lane 1, control; lane 2, 1 μg/ml; lane 3, 10 μg/ml; lane 4, 100 μg/ml; lane 5, 1000 μg/ml. B) Densitometric analysis of autoradiogram. Doses were 1, 10, 50, 75, 100, 1000 μg/ml. Each value (%) represents the average of densitometric analyses of autoradiogram from three experiments.

purified transcription factors from HeLa cell nuclear extract as a standard system. Figure 1 shows the dose-response autoradiogram and densitometric analysis of the autoradiogram based on the assay in the presence of **1**. At concentrations lower than 10 μg/ml, **1** did not affect the transcription activity. However, **1** inhibited the activity at more than 50 μg/ml. This result is interesting, since the concentration of **1** was the same as that for the inhibition of *in vitro* microtubule polymerization.^{7a)} This experiment was performed on the assumption that the inhibition of transcription by **1** may be due to interaction with transcription factors and/or RNA polymerase II. Next, we examined the effect of **1** on RNA polymerase II activity assayed by the random incorporation of ribonucleotides, in which denatured salmon sperm DNA was used as a template in the presence of MnCl₂ as a divalent cation. As shown in Table I, **1** had inhibitory activity on mammalian RNA polymerase II itself. The activity of **1** on RNA polymerase II itself was weaker than that on the transcription at 100 μg/ml.

Consequently, we examined the effects of *Z,Z*-dienestrol,

TABLE I. Effects of Compounds (**1**–**6**) on RNA Polymerase II Activity

Concentration (μg/ml)	Compounds	Inhibition (%)
1	1	16
	2	0
	3	0
	4	0
	5	0
	6	0
10	1	36
	2	3
	3	12
	4	0
	5	0
	6	0
100	1	55
	2	24
	3	62
	4	0
	5	0
	6	0

Each value represents the average of three experiments.

Fig. 2. Effects of Drugs on Transcription by RNA Polymerase II

Each value (%) represents the average of densitometric analyses of autoradiogram from three experiments. ○, diethylstilbestrol (1); ■, Z,Z-dienestrol (2); □, E,E-dienestrol (3); ▲, indenestrol A (4); △, indenestrol B (5); ●, estradiol (6).

indenestrol A, E,E-dienestrol, and indenestrol B on *in vitro* transcription activity. As shown in Fig. 2, E,E-dienestrol (3) and indenestrol B (5) gave higher inhibitory activity than Z,Z-dienestrol (2) and indenestrol A (4), respectively. Next, we examined the effects of the drugs on RNA polymerase II itself, as seen in Table I. These two kinds of experiments indicated that 2 and 3 inhibited not only the transcription activity but also RNA polymerase II activity as is the case with microtubule polymerization. Moreover, the result show that indenestrols (4 and 5) had an inhibitory effect only on the transcription factors, since inhibitory activities of the indenestrols on RNA polymerase II itself were not observed.

We also tested the effect of estradiol (6), the most potent naturally occurring estrogen in mammals. However, in spite of the reported inducibility of mitotic non-disjunction by 200 μ M (54.4 μ g/ml) estradiol in HeLa cells,²⁴ the results of turbidity measurement and electron microscopy have indicated that it has no ability to interact with microtubules or microtubule protein *in vitro*.^{7a)} On the other hand, we previously discovered that estradiol had toxic activity on Chinese hamster V79 cells, and inhibited microtubule polymerization in cultured V79 cells.²⁵ Therefore, we examined the effects of estradiol (6) on transcription by mammalian RNA polymerase II and on RNA polymerase II itself. As seen in Fig. 2 and Table I, 6 did not affect the activities of transcription as well as RNA polymerase II itself. These results suggest that the target of the inhibitory activity of estradiol was neither general transcription factors nor RNA polymerase II itself.

As part of our series of studies aimed at achieving a better understanding of the mechanism of action of tumorigenic

stilbene estrogens, the direct effects of diethylstilbestrol (1) and its analogues and estradiol (6) on the transcription activity of mammalian RNA polymerase II were clarified in the present investigation. Present clear-cut results would not be obtained if a nuclear extract was used as an assay system.

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