Effects of 7, 12-Dimethylbenz [a] Anthracene on RNA Polymerase in Isolated Mammary Gland Cell Nuclei[1] (35343)

TAKESHI TOMINAGA, THOMAS L. DAO, AND PAUL R. LIBBY

Department of Breast Surgery, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, New York 14203

Libby and Dao (1) demonstrated that the incorporation of sodium ^{14}C-formate into mammary gland RNA in female rats decreased within 24 hr after a single feeding of 20 mg of 7,12-dimethylbenz[*a*]anthracene (DMBA). In males, there was a gradual increase in the incorporation. In castrated rats, the decrease or increase in ^{14}C-formate incorporation is dependent on the presence of an estrogen or androgen, respectively. In contrast, Tominaga *et al.* (2) showed that ^{3}H-thymidine incorporation into mammary gland DNA was inhibited in both female and male rats receiving DMBA. Since administration of DMBA induces mammary cancer invariably in female rats but rarely in males, we believe that the early biochemical events occurring in the process of carcinogenesis induced in the mammary gland by DMBA may be related to the effects of the carcinogen on RNA synthesis. The present paper reports data from our experiments dealing with the effects of DMBA on RNA polymerase activity in isolated mammary gland cell nuclei.

Materials and Methods. Sprague-Dawley rats (60 to 65 days old) from the Holtzman Company, fed on a conventional ration (Rockland diet) and given water *ad libitum*, were fed 20 mg of DMBA in 1 ml of olive oil. Rats were killed by cervical dislocation 1, 2, or 4 days after feeding of the carcinogen. After removal of the lymph nodes, the abdomino-inguinal mammary glands from 4 rats were excised, and were pooled in ice-cold isotonic saline. The isolation of cell nuclei from the mammary gland tissue was carried out by a modification of the method described by Widnell and Tata (3, 4). Pooled mammary glands were minced with scissors, and were homogenized in a Virtis homogenizer in 20 vol of 0.25 *M* sucrose containing 3 m*M* $MgCl_2$ and 0.3% Tween 20. Homogenization was carried out at 0° at 100 V two times, each for 30 sec, separated by a 1-min interval. We added Tween 20 to the homogenizing medium and centrifuged at low speed to allow the separation of released nuclei from the fat in the mammary gland tissue.

The homogenate was filtered through 8 layers of gauze and centrifuged at 1500*g* for 10 min. The pellet was resuspended in 0.25 *M* sucrose containing 1 m*M* $MgCl_2$, using 5 strokes of a Teflon pestle (clearance 0.004–0.006 in.), filtered through 4 layers of gauze, and centrifuged at 700*g* for 10 min. The pellet was washed with the same medium and then suspended in 1 to 2 ml of this medium for use as an enzyme source.

For assay of Mg^{2+}-activated RNA polymerase, the incubation mixture contained 100 μmoles of Tris–HCl buffer (pH 7.4), 1 μmole of $MgCl_2$, 10 μmoles of cysteine, 10 μmoles of NaF, 1 μmole of ATP, 0.4 μmole each of GTP and CTP, 2 mμmole of TP and 4 μCi of ^{3}H-UTP (sp act 1.78 Ci/mmole), and 0.2 ml of enzyme solution (0.14 to 0.4 mg of DNA) in a total volume of 1.0 ml. Cysteine and ATP solutions were adjusted to pH 7.4 with NaOH.

For assay of Mn^{2+}-$(NH_4)_2SO_4$-activated RNA polymerase activity, the incubation mixture contained 100 μmoles of Tris–HCl buffer (pH 8.6), 4 μmoles of $MnCl_2$, 1 μmole of ATP, 0.4 μmole each of GTP and CTP, 2 mμmole of UTP, 2 μCi of ^{3}H-UTP and 0.1 ml of saturated $(NH_4)_2SO_4$ solution, and 0.2 ml of the enzyme solution in a total volume of 1.0 ml. The saturated $(NH_4)_2SO_4$ solution

[1] This study was supported by Grant CA-04632-11 from the National Cancer Institute, Institutes of Health.

and the ATP solution were adjusted to pH 8.6 with NH_4OH and NaOH, respectively.

Both incubations were carried out for 10 min at 37°. Duplicates of each homogenate were run in each experiment. At the end of the incubation, the tubes were chilled in ice, and 3 ml of 10% trichloroacetic acid (TCA) containing 0.2 mole of UTP were added. The tubes were centrifuged, and the precipitate was washed three times with 3 ml of 5% TCA containing 0.2 μmoles of UTP and once with 95% ethanol. Next the precipitate was suspended in 1 ml of 0.3 NaOH and incubated for 16 hr at 37°. The suspension was then chilled in ice, and 0.1 ml of cold concentrated perchloric acid was added. After centrifugation of the mixture, 0.5 ml of the supernatant was added to 15 ml of scintillation fluid, and radioactivity was counted with a liquid scintillation counter (Nuclear Chicago, 720 series). One liter of scintillation fluid was made of 40 ml of Liquifluor (Nuclear Chicago Corp., Des Plaines, Ill.), 80 g of naphthalene, 560 ml of toluene, and 400 ml of ethylene glycol monomethyl ether. The precipitate containing DNA was washed twice with cold 5% TCA and incubated with 1.5 ml of 5% TCA for 15 min at 90°. After centrifugation, the DNA content of the supernatant was determined (5). Zero-time control values were determined for each experiment, and were subtracted from the values for the incubated tubes. The activity of RNA polymerase was expressed as amount of UTP (μμmoles) incorporated into RNA per milligram of DNA in 10 min.

Results. Examination of the nuclear fractions by phase contrast microscopy revealed contamination with cytoplasmic substances, but it never amounted to more than 5% of the nuclei. The RNA/DNA ratio of the nuclear fraction ranged from 0.25 to 0.45 for control male and female rats, and was not significantly affected by prior feeding of DMBA. Inclusion of Tween 20 was essential for the preparation of a good nuclear fraction.

The enzyme activities in rat mammary cell nuclei exhibited many of the characteristics of RNA polymerase already described (6). Both activities were dependent on the presence of all four nucleotides. Both enzyme

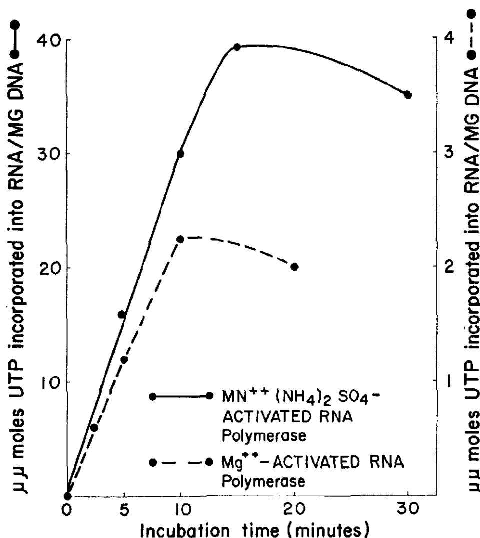

FIG. 1. Rate of synthesis of Mn^{2+}-activated RNA polymerase in mammary cell nuclei in relation to time of incubation.

activities were linear with enzyme concentration up to 0.4 mg of DNA added, and were linear with time of incubation up to 10 min (Fig. 1). The Mg^{2+}-activated enzyme required the presence of cysteine for full activity. The requirement of the enzymes for divalent metal ions was similar to that reported by Widnell and Tata (6), but we found optimum activation of the Mg^{2+}-activated enzyme to occur at 10^{-3} *M* $MgCl_2$, rather than at the 4×10^{-3} *M* concentration reported by these authors.

A striking difference was found, however, in the pH curves of the two activities. Widnell and Tata (6) reported that the Mg^{2+}-activated enzyme was most active at about pH 8.5, and that the Mn^{2+}- and $(NH_4)_2SO_4$-activated enzyme was most active at about pH 7.5. Figure 2 illustrates the curves found for the mammary gland enzymes when the effect of pH was tested. It is evident that the pH relationships are approximately the opposite of those found for the liver enzymes by Widnell and Tata (6).

The effects of pretreatment of the rats with DMBA on RNA polymerase activity are shown in Table I. The rats in the experimental groups were fed 20 mg of DMBA on 1, 2, or 4 days previous to sacrifice. Nuclei were

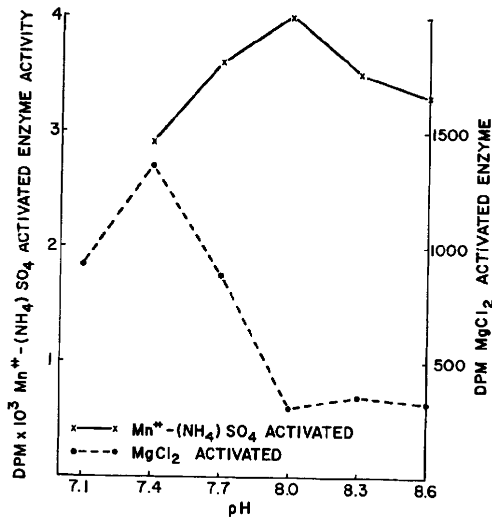

FIG. 2. The pH relationship of Mn^{2+}-activated RNA polymerase activity in the rat mammary gland cell nuclei.

assayed for the two polymerase activities in these animals, and the results were compared with those for the control animals. In female rats, the enzyme activated by Mn^{2+}-ammonium sulfate showed inhibition in relation to control values at 1 and 2 days after feeding. At 4 days after feeding, there was a significant rise above the control values. In male animals, the values for the experimental group did not differ significantly from the values for the control group. The result also showed that there was no change in the Mg^{2+}-activated enzyme activities before and after DMBA treatment. However, there was a great spread in the measurements, and the results were less significant.

Discussion. The two enzyme species studied in the present investigation correspond in many respects to the activities that have previously been mentioned in the literature. Widnell and Tata (6) first suggested, on the basis of differential ion activation and differences in response to changes in pH, that two different enzymes were involved. Later work indicated that the two activities were differentially localized in the nucleus (7), and Roeder and Rutter (8) succeeded in solubilizing and chromatographically separating the two enzymes. These authors further demonstrated that one chromatographic spe-

TABLE I. Effect of DMBA on DNA-Dependent RNA Polymerase Activities of the Mammary Cell Nuclei.[a]

Isolated mammary cell nuclei prepared from four pairs of mammary glands from four rats were incubated with ^3H-UTP in the presence of all four ribonucleoside phosphates. In DMBA-treated groups, rats were given 20 mg of the carcinogen by oral feeding. Results are expressed as micromicromoles of UTP incorporated into RNA/10 min/mg of DNA. All experiments were repeated twice and results were similar.

After DMBA	Mg^{2+}-activated RNA polymerase				Mn^{2+}-$(NH_4)_2SO_4$-activated RNA polymerase			
	♀		♂		♀		♂	
(days)	Control	DMBA	Control	DMBA	Control	DMBA	Control	DMBA
1	2.29 ± 0.91	1.74 ± 0.14	0.97 ± 0.17	1.63 ± 0.52	36.46 ± 7.60	25.02 ± 3.59[b]	27.66 ± 3.35	26.13 ± 2.56
2	1.39 ± 0.34	1.74 ± 0.17	1.73 ± 0.22	1.46 ± 0.09	29.09 ± 2.79	22.52 ± 4.56[b]	28.92 ± 6.46	25.48 ± 1.46
4	2.00 ± 0.17	2.35 ± 0.34	1.60 ± 0.10	1.78 ± 0.09	34.70 ± 2.92	46.88 ± 2.63[c]	33.32 ± 4.09	29.77 ± 2.08

[a] All p values were calculated by Student's t test; from control: [b] $p < 0.05$; [c] $p < 0.001$.

cies was localized in the nucleolus, and the other in the nucleoplasm (9). Unfortunately, they ran all of their assays at one pH value for both enzymes, and have not reported pH curves for the solubilized, partially purified enzymes from either rat liver or sea urchin embryos. The results of the study reported in the present paper suggest that the enzymes in the rat mammary gland are distinct from the activities reported for the rat liver by Widnell and Tata (6). RNA polymerases have also been examined in other rat tissues, such as those of the uterus (10) and prostate (11). In these latter studies, both enzyme activities were assayed at one pH. This question may only be resolved by studies on the two enzymes from various tissues.

The effect of DMBA on the mammary gland polymerase activities is of great interest because of a good correlation between the effect on the RNA polymerase activities and the effect of DMBA on RNA synthesis *in vitro* (1). We (Libby and Dao, unpublished data) have found that if minces from a mammary gland are incubated with labeled adenosine at various times after feeding DMBA, an inhibition of label incorporation occurs which is strikingly similar to the inhibition of Mn^{2+}-activated RNA polymerase as reported in the present paper. We also observed in this study an increase in the Mg^{2+}-activated polymerase in male rats 1 day after DMBA feeding. This finding parallels the earlier finding of a rise in adenosine incorporation in the male mammary gland 1 day after DMBA feeding.

Because the effect of DMBA on RNA synthesis is specific for the female mammary gland, which develops tumors, and not for the male mammary gland, which does not develop tumors, it appears that some effect on RNA synthesis (whether overall or for a specific molecular species is not known) may be related to the carcinogenic effect of DMBA.

Summary. A method for isolation of mammary gland cell nuclei was described. DNA-dependent RNA polymerase activity in isolated mammary gland nuclei was measured in rats fed a single dose of DMBA. In female rats, there was a significant inhibition of Mn^{2+}–$(NH^4)_2SO_4$-activated RNA polymerase activity within 24 hr after the feeding of DMBA. Inhibition continued for 2 days, but thereafter, the enzyme activity rose significantly above the control value. In contrast, RNA polymerase activity was not depressed in male rats treated with DMBA.

1. Libby, P., and Dao, T. L., Science 153, 303 (1966).

2. Tominaga, T., Libby, P., and Dao, T. L., Cancer Res. 30, 118 (1970).

3. Widnell, C. C., and Tata, J. R., Biochem J. 92, 313 (1964).

4. Widnell, C. C., Hamilton, T. H., and Tata, J. K., J. Cell. Biol. 32, 766 (1967).

5. Schneider, W. C., Methods Enzymol. 3, 680 (1957).

6. Widnell, C. C., and Tata, J. R., Biochim. Biophys. Acta 87, 531 (1964).

7. Widnell, C. C., and Tata, J. R., Biochim. Biophys. Acta 123, 478 (1966).

8. Roeder, R. G., and Rutter, W. J., Nature (London) 224, 234 (1969).

9. Roeder, R. G., and Rutter, W. J., Proc. Nat. Acad. Sci. U.S.A. 65, 675 (1970).

10. Gorski, J., J. Biol. Chem. 239, 889 (1964).

11. Hancock, R. L., Felis, R. F., Shaw, M., and William-Ashman, H. G., Biochim. Biophys. Acta 55, 257 (1962).

Received Sept. 1, 1970. P.S.E.B.M., 1971, Vol. 136.