Effects of Dimethylnitrosamine on RNA Synthesis and Metabolism in Mouse Liver

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ABSTRACT

Following i.p. injection of dimethylnitrosamine into male C57BL mice, synthesis of liver nuclear heterogeneous RNA was inhibited significantly, reaching approximately 20% of control within 2 hr of a dose of 40 mg/kg. Synthesis of nucleolar RNA was also inhibited, although to a smaller extent, reaching about 70% of control after the same treatment. These effects were observed both during RNA synthesis in vivo and during in vitro transcription with isolated nuclei and nucleoli. Examination of RNA polymerases I and II, isolated and partially purified by diethylaminoethyl Sephadex column chromatography, did not indicate any change either in their activities in the transcription of exogenous DNA or in their in vivo binding to chromatin. On the other hand, the activity of purified chromatin as a template for transcription by added, partially purified RNA polymerase II was significantly reduced, suggesting that carcinogen-induced damage to chromatin was the cause of the observed inhibition of heterogeneous RNA synthesis. When purified DNA was used in place of chromatin as a template for transcription by partially purified RNA polymerase II, no inhibition was observed.

Dimethylnitrosamine treatment had a pronounced effect on the kinetics of appearance of the cytoplasmic RNA species. Four hr after a 40-mg/kg dose of dimethylnitrosamine, the rate of appearance in the cytoplasm of polyadenylate-containing RNA was inhibited by 50%, while that of 4S, 18S, and 28S ribosomal RNA was inhibited by over 80%.

INTRODUCTION

Among the early effects which follow the administration of a single dose of the hepatocarcinogen DMN to rats or mice is a rapid inhibition of liver protein and RNA synthesis (9, 10, 35, 36). Whereas the mechanism of the inhibition of protein synthesis has been the subject of extensive studies (21–23), the corresponding effects on RNA synthesis are not so well understood. In an early study, Steward and Magee (35) found that total nRNA synthesis in rat liver is inhibited following injection of DMN (40 mg/kg), maximal inhibition being about 40% 9 hr after administration of the carcinogen. In a later study, Herzog and Farber (9) reported a similar result and attributed the inhibition to a decrease in the activity of the DNA-dependent RNA polymerases I and II, rather than to any alteration in the template activity of chromatin. Finally, Hultin et al. (10) reported partial inhibition of nRNA synthesis in the liver of mice 90 min after treatment with DMN (36 mg/kg), without, however, attempting to establish the molecular mechanism of this inhibition.

nRNA synthesis includes the synthesis of a number of classes of RNA, the main ones being hnRNA and nucleolar RNA. The former, following various stages of postsynthetic modification, gives rise to mRNA and is transported to the cytoplasm, while nucleolar RNA, also after processing, appears in the cytoplasm as mature rRNA. Any study of the effects of DMN on RNA synthesis should ideally examine separately the synthesis, as well as the processing and transport, of each of the various species of nRNA. That carcinogens (including DMN) cause changes in the processing and transport of RNA is well established (25, 26, 33). Thus, even a single administration of DMN causes the appearance in the cytoplasm of new RNA sequences not normally found there. This change is accompanied by a partial release from the energy requirements for RNA transport from the nucleus as studied in vitro (31, 34).

In view of the importance of transcription and RNA processing and transport in the control of gene expression, we have set out to examine in detail the effects of DMN on the corresponding processes for various RNA species. We have used for this purpose C57BL mice because the rapid metabolism of the carcinogen by these animals permits the examination of its very early effects, prior to the onset of any acute necrotic effects on the liver.

MATERIALS AND METHODS

Treatment of Animals. The animals used were male C57BL mice, 10 to 12 weeks old, weighing 25 to 30 g each, obtained from the Thessaloniki Cancer Institute, Thessaloniki, Greece. The synthesis of RNA was examined after the administration of a single i.p. injection of DMN (Aldrich Chemical Co., Dorset, United Kingdom) dissolved in 0.9% NaCl solution (saline) at a concentration of 10 mg/ml or, in the case of control animals, of 0.1 ml saline. Four hr (unless otherwise indicated) after the administration of DMN, [3H]thymidine (usually 25 to 30 Ci/mmol; The Radiochemical Centre, Amersham, United Kingdom; 91 Ci/10 g body weight dissolved in saline) was injected i.p., usually for 30 min. After this time, the animals were killed with diethyl ether, and the livers were rapidly removed and frozen in liquid nitrogen, unless processed immediately.

Measurement of hnRNA and Nucleolar RNA Synthesis in Vitro. As a measure of the synthesis of each RNA species, we used its specific radioactivity, i.e., the amount of [3H]radioactivity incorporated per µg of RNA after a given pulse ([3H]thymidine).

hnRNA was isolated from liver nuclei according to the method of Nissling and Sekeris (20). The livers were homogenized in 2 volumes of TSS buffer and filtered in succession through 2 and 6 layers of gauze. After centrifugation at 800 × g for 5 min, the cytoplasmic supernatant was used (where appropriate) for the preparation of cytoplasmic RNA (see below), and the crude nuclear pellet was homogenized in an all-glass homogenizer with 2-volumes of 2.2 M sucrose in TSS and centrifuged at 40,000 × g for 90 min. The purified nuclei thus obtained were washed once with 50 mM EDTA in TSS and then used for the extraction
of the hnRNP. For this purpose, nuclei were incubated at 4° with 0.14 M NaCl: 1 mM MgCl₂: 10 mM Tris-HCl, pH 8.0 (1 ml/mouse liver) twice for 30 min each time, with a centrifugation at 800 x g for 10 min in between. Following the second incubation, the mixture was sonicated using an MSE sonicator (full amplitude, exponential probe, 6 cycles of 10 sec each) and centrifuged at 10,000 x g for 10 min. The 3 supernatant extracts thus obtained were pooled and used as the hnRNP extract for the preparation of hnRNA.

hnRNA was isolated from the hnRNP extract by means of four 10-min extractions with an equal volume of a 1:1 mixture of Kirby phenol (500 ml redistilled phenol, 55 ml water, 70 ml redistilled m-cresol, 0.5 g 8-hydroxyquinoline, saturated with hnRNP extraction buffer) and chloroform:isoamyl alcohol (24:1). In the presence of 1% SDS and 10 mM EDTA. After each extraction, the aqueous phases were mixed, and the hnRNA was finally precipitated with 2.5 volumes of ethyl alcohol in the presence of 0.1 volume of 0.1 M sodium acetate. It was washed consecutively with 3 M sodium acetate (2 times), 70% ethyl alcohol, 95% ethyl alcohol, absolute ethyl alcohol, and diethyl ether and dried under N₂.

Nuclease RNA was extracted from isolated nuclei as described by Muramatsu and Onishi (18). Nuclei were suspended in 0.34 M sucrose:0.05 mM MgCl₂, sonicated in an MSE sonicator (16 um amplitude, exponential probe, 4 cycles of 15 sec each) and centrifuged over 20 mg of sucrose:0.05 mM MgCl₂ at 2000 x g for 20 min. The nuclease pellet was further purified by a second, similar centrifugation through 0.88 mg sucrose:0.05 mM MgCl₂ and used for the extraction of nuclease RNA. Following mild homogenization in 0.14 M NaCl:0.1 M sodium acetate:0.3% SDS, pH 5.10 (10 ml/mouse liver), an equal volume of Kirby phenol was added, and the mixture was homogenized briefly at room temperature and then shaken well, first at 65° and then at room temperature for 15 min each time. The aqueous phase was reextracted for 10 min at room temperature successively first with an equal volume of phenol and then with 1:1 mixture of phenol and chloroform:isoamyl alcohol (24:1). Finally, the RNA was precipitated from the aqueous phase and washed as described for hnRNA. For the measurement of the specific radioactivity, hnRNA and nuclease RNA were dissolved in a 1:2 dilution of binding buffer, the bound RNA was eluted with 1 mM EDTA:0.05% SDS at pH 6.5, and its specific radioactivity was measured as described above.

In Vitro RNA Synthesis with Isolated Nuclei and Nucleoli. In vitro RNA synthesis utilizing isolated nuclei was carried out by the method of Marzulli et al. (17). To 50-μl mixtures containing 5 mM magnesium acetate; 10 mM MnCl₂; 80 mM or 200 mM (NH₄)₂SO₄ (see below); 0.4 mM concentrations each of ATP, GTP, and CTP; 0.04 mM UTP; and 1.67 μCi [5,6-³H]UTP (45.5 Ci/mmol; The Radiochemical Centre) were added 10 μl of a suspension of nuclei (usually containing 3 x 10⁶ nuclei) in TGME buffer containing 0.1 mM PMSF. Transcription was carried out at 30° for 5 to 30 min in the presence of low (80 μM) or high (200 μM) concentrations of (NH₄)₂SO₄ as well as in the absence or presence of α-amanitin (1 μg/ml) for the determination of RNA polymerases I + III or RNA polymerase II activities. The reaction was stopped by the addition of 50 μl of 50 mM Na₂PO₄:1% SDS and pH 7.5, and its specific radioactivity was measured as described above.

In vitro RNA synthesis with isolated nuclei was carried out in a similar manner, except that only 80 μM (NH₄)₂SO₄ was used in the absence of α-amanitin. Nuclei were suspended in 0.88 mg sucrose:50% glycerol (v/v); 10 mM Tris-HCl (pH 7.5) prior to addition to the reaction mixtures.

Examination of nRnase Activity. The total RNase activity of purified nuclei or of solubilized extracts of nRNA polymers was examined using [³H]poly(U) as substrate as described by Nygard and Hultin (24). Briefly, 3 x 10⁶ dpm [³H]poly(U) (1.63 mCi/mg; The Radiochemical Centre) were added 10 μl of the reaction mixtures, which had been equilibrated with binding buffer. After exhaustive washing with binding buffer, the bound RNA was eluted with 1 mM EDTA:0.05% SDS:10 mM Tris-HCl (pH 7.5). The poly(A)-containing RNA obtained was purified further by means of a second similar cycle of chromatography, and its specific radioactivity was measured as described above.

Measurement of rRNA Synthesis. Total cytoplasmic RNA was extracted from the first supernatant obtained after homogenization for the preparation of nuclei as described in the previous section. Heperin and Triton X-100 were added at final concentrations of 50 μg/ml and 1%, respectively, and the mixture was centrifuged at 5000 x g for 10 min (7). After addition of EDTA and SDS to the supernatant at final concentrations of 10 mM and 1%, respectively, the mixture was extracted for 10 min with an equal volume of a 1:1 mixture of Kirby phenol and chloroform:isoamyl alcohol (24:1). The aqueous phase was extracted for at least 3 more times in the same way until no interphase could be seen, and the RNA was precipitated and washed as described earlier for nRNA. The 3 species of rRNA (4S, 18S, and 28S) were prepared by chromatography on oligodeoxythymidylic acid-cellulose (Sigma) (7). The RNA was dissolved in 1 ml of binding buffer (0.5 M NaCl:1 mM EDTA:0.05% SDS:10 mM Tris-HCl, pH 7.5), diluted with 9 ml of dimethyl sulfoxide and 1 ml of 1 M LiCl:50 mM EDTA:2% SDS:100 mM Tris-HCl (pH 6.5), and denatured by heating to 65° for 5 min. The mixture was then diluted 10 times with binding buffer and passed through a column of oligodeoxythymidylic acid-cellulose (1 ml packed bed volume) which had been equilibrated with binding buffer. After exhaustive washing with binding buffer, the bound RNA was eluted with 1 mM EDTA:0.05% SDS:10 mM Tris-HCl (pH 7.5). The poly(A)-containing RNA obtained was purified further by means of a second similar cycle of chromatography.

Three hundred units of total RNA polymerases was extracted from nuclei by suspension of the latter in 0.14 M sucrose:1 mM MgCl₂:6.25 mM NaF:2.98 mM α-mercapto-ethanol:0.1 mM (NH₄)₂SO₄:0.63 mM concentrations each of ATP, GTP, CTP, and UTP:0.01 mM UTP:1.56 μCi [5,6-³H]UTP (45.5 Ci/mmol); 50 mM Tris-HCl (pH 8.0); 20 μg calf thymus DNA:40 μl RNA polymerase solution. Where necessary, α-amanitin (1 μg/ml) was included. Incubation was carried out at 30° for various time intervals, after which it was interrupted, and the extent of RNA synthesis was measured as described above for transcription with isolated nuclei.

Measurement of Chromatin-bound and Free RNA Polymerases. Chromatin-bound and free RNA polymerases were separately solubilized and measured according to the method of Yu (38). The free form of the polymerases was extracted from nuclei by suspension of the latter in 0.34 mg sucrose:10 mM MgCl₂:25 mM KCl:50 mM Tris-HCl (pH 7.80) for 10 min at 4°. After centrifugation of the suspension at 700 x g for 10 min, the chromatin-bound RNA polymerases were extracted from the
nuclear pellet as described in the previous section for total RNA polymerase extraction. Where appropriate, the separately solubilized RNA polymerases were partially purified by DEAE-Sephadex column chromatography as already described.

Isolation and Transcription of Chromatin. Chromatin was prepared from purified nuclei by the method of Beato et al. (1). The nuclei were washed successively twice with 75 mM NaCl: 24 mM EDTA and twice with 50 mM Tris-HCl (pH 8.0) at 4°C. Each washing was followed by centrifugation at 10,000 g for 15 min except for the last one, after which the mixture (chromatin) was centrifuged through 1.7 M sucrose: 50 mM Tris-HCl (pH 8.0). The pellet of purified chromatin was redissolved in 50 mM Tris-HCl, pH 8.00, homogenized briefly in a Vir-Tis homogenizer, and finally centrifuged at 80,000 g for 10 min. The supernatant was used for RNA synthesis with or without exogenously added RNA polymerase II partially purified by DEAE-Sephadex chromatography as already described. For RNA synthesis utilizing isolated chromatin, the procedure used was as described in the previous section for the estimation of the activity of RNA polymerases, except that no calf thymus DNA was included. The exogenous RNA polymerase added had been concentrated by ultrafiltration, and sufficient RNA polymerase was included to give a net activity at least 3 times higher than that observed with the endogenous, chromatin-bound enzyme.

Preparation and In Vitro Transcription of DNA. DNA was prepared from mouse liver by the method of Marmur (16), which involved extraction of nuclei (dissolved in 0.1 M NaCl: 5 mM EDTA: 0.5 M NaClO4: 1% SDS: 10 mM Tris-HCl, pH 7.90) twice with chloroform:isoamyl alcohol (24:1), treatment of DNA with RNase A and proteinase K, 2 further extractions with chloroform:isoamyl alcohol, one extraction with Kirby phenol, and precipitation with ethyl alcohol.

Transcription in vitro of purified DNA was carried out under conditions which permitted the separate examination of the number of initiation sites, the rate of formation of initiation complexes, and the rate of elongation of the RNA chains as described by Cedar and Felsenfeld (5). In all cases, initiation was carried out in the presence of 3 of the nucleotides, and elongation was subsequently allowed to proceed under conditions of high ionic strength [0.4 M (NH4)2SO4] which prevented reinitiation. RNA polymerase II partially purified by DEAE-Sephadex chromatography and concentrated by ultrafiltration was used.

Initiation of transcription was carried out by incubation, in a total volume of 500 μl, of 1 mM MnCl2: 0.08 mM concentrations each of ATP and GTP; 6 μCi [5,6-3H]UTP (45.5 Ci/mmol); 10 mM Tris-HCl (pH 7.90), and appropriate amounts of DNA dissolved in water and RNA polymerase II extract at 37°C. After the appropriate period, elongation without reinitiation was permitted to occur by addition of the fourth nucleotide CTP (final concentration, 60 mM), MnCl2 (3 mM), and (NH4)2SO4 (0.4 M). Chain elongation was permitted to proceed for the required length of time, after which it was stopped by the addition of 10 μl of 0.25 M Na2P2O7: 5% SDS, the mixture was spotted on DE81 filters, and the amount of RNA synthesis that had occurred was measured as described above.

For measurement of the rate of formation of the initiation complexes, the mixture contained (4.6 μg DNA and a fixed amount of RNA polymerase II empirically found to give satisfactory amounts of RNA synthesis) were incubated for 0 to 20 min, while elongation was permitted to proceed in each case for 15 min. For comparison of the number of initiation sites in DNA from control and DMN-treated animals, initiation was carried out for 15 min using a fixed quantity of RNA polymerase II and varying amounts of DNA (0 to 4.8 μg) with an elongation time of 15 min. Finally, for the measurement of the rate of chain elongation, a fixed amount of DNA (1 μg) and a fixed amount of RNA polymerase II sufficient to saturate the DNA (empirically obtained from the curve of the titration of RNA polymerase II with DNA) were incubated in initiation mixtures for 15 min, after which elongation was permitted to proceed for varying lengths of time (0 to 20 min).

Measurement of Protein and DNA. Concentration of protein was measured by the method of Bradford (2). DNA was estimated colorimetrically by the method of Burton (3) or, when necessary, by the microfluorometric procedure of Kisse and Robins (11) as modified by Setaro and Morey (32).

RESULTS

Synthesis of Heterogeneous and Nucleolar RNA in Vivo.

Following a single injection of DMN, there is a rapid inhibition of liver hnRNA synthesis (Chart 1). This inhibition is dose related (Chart 2) and, at the highest dose examined (40 mg/kg), reaches approximately 80% within 2 hr (Chart 2, inset).

The kinetics of the incorporation of 3H radioactivity from [3H]orotic acid into nuclear RNA (Chart 3) indicates that the rate of synthesis of this species of RNA is also reduced by DMN.
Itself inhibits the activity of RNA polymerase II. The results of Table 2 indicate 31% inhibition of nucleolar RNA synthesis (i.e., activity, either in control or in DMN-treated animals. The above fig DNA per assay mixture) did not reveal any detectable RNase of nRNA polymerases (equivalent to up to 2 x 10^7 nuclei or 120 µg DNA per assay mixture) did not reveal any detectable RNase activity, either in control or in DMN-treated animals. The above results demonstrate that the observed reduction in RNA synthesis is neither an artifact resulting from the activation by DMN of nuclear nucleases nor a consequence of changes in the nucleotide pool size and confirm the results of Huitlin et al. (10), who measured the nucleotide pool sizes directly and found them to be unaltered after DMN treatment.

The effect of DMN on nucleolar RNA synthesis was further examined separately in an analogous in vitro assay system utilizing isolated liver nucleoli. The results obtained by this assay (Table 2) indicate 31% inhibition of nucleolar RNA synthesis (i.e., although to a smaller degree than for hnRNA.

**RNA Synthesis in Vitro.** The effects of DMN on the rate of synthesis of RNA were also examined in a series of in vitro assays. In the first of these, whole nuclei isolated from the livers of control or DMN-treated animals were used to carry out in vitro transcription utilizing various ionic conditions so as to enhance selectively the expression of RNA polymerases I + III or II. Furthermore, transcription was carried out in the absence or presence of α-amanitin at a concentration (1 µg/ml) that selectively inhibits the activity of RNA polymerase II. The results of these experiments (Table 1) show that RNA polymerase II activity is inhibited by 70% while the activity of RNA polymerases I + III is inhibited only by 28%, in agreement with the results obtained in vivo. It should be noted that incubation of [3H]poly (U) with nuclei (up to 10^6 nuclei per assay mixture) or solubilized extracts of nRNA polymerases (equivalent to up to 2 x 10^7 nuclei or 120 µg DNA per assay mixture) did not reveal any detectable RNase activity, either in control or in DMN-treated animals. The above results demonstrate that the observed reduction in RNA synthesis is neither an artifact resulting from the activation by DMN of nuclear nucleases nor a consequence of changes in the nucleotide pool size and confirm the results of Huitlin et al. (10), who measured the nucleotide pool sizes directly and found them to be unaltered after DMN treatment.

The effect of DMN on nucleolar RNA synthesis was further examined separately in an analogous in vitro assay system utilizing isolated liver nucleoli. The results obtained by this assay (Table 2) indicate 31% inhibition of nucleolar RNA synthesis (i.e., RNA polymerase II activity), in reasonable agreement with the results obtained by the other assays.

**Examination of Activities of the RNA Polymerases.** In order to examine the cause of the reduction in the rate of RNA synthesis brought about by DMN, the nuclear DNA-dependent RNA polymerases were solubilized by treatment of nuclei with 0.3 M ammonium sulfate according to the method of Roeder and Rutter (29). The extract thus obtained was used to carry out transcription of exogenously added calf thymus DNA in the absence or presence of α-amanitin. The results, shown in Table 3, indicate clearly that the extracts obtained from control and DMN-treated animals exhibit similar activity levels for RNA polymerases I + III and II. This suggests that no damage had been caused by the carcinogen to the transcribing enzymes themselves.

The activity of the RNA polymerases was further examined after partial purification by DEAE-Sephadex column chromatography. Table 4 shows the results obtained, which confirm that the activities of the enzymes themselves are not significantly affected by the carcinogen.

**RNA Synthesis with Isolated Chromatin.** In view of the above

![Graph](image)
results, the template activity of chromatin was subsequently examined. After isolation and purification, chromatin was used as a template for RNA synthesis utilizing exogenously added RNA polymerase II obtained from the livers of control or DMN-treated animals and partially purified by DEAE-Sephadex column chromatography as described in "Materials and Methods." The results obtained (Table 5) show clearly that chromatin isolated from DMN-treated animals shows significantly reduced template activity for transcription by RNA polymerase II, regardless of whether the latter came from control or DMN-treated animals. Furthermore, the extent of inhibition of the template activity of chromatin (approximately 75%) is almost identical with that observed for hnRNA synthesis in vivo, suggesting that the latter is probably entirely due to the impaired function of chromatin.

RNA Polymerase Binding to Chromatin In Vivo. RNA polymerase is believed to exist in vivo in equilibrium between 2 functional forms, one free and the other bound to chromatin (38). This equilibrium has been shown to be shifted by a number of carcinogens in favor of the free form of RNA polymerase II, i.e., the binding of the latter to chromatin is reduced following carcinogen treatment (8, 39, 41). The possibility that such an effect may be responsible for the observed inhibition of RNA synthesis was examined by isolating and estimating separately the free and chromatin-bound forms of the RNA polymerases (38). Following separate solubilization of the 2 forms of RNA polymerases, the extracts were subjected to DEAE-Sephadex column chromatography, and the activities thus separated were measured. As shown in Table 6, no significant changes in the proportions of free and chromatin-bound RNA polymerases are induced by DMN treatment.

RNA Synthesis with Isolated DNA. Having established that the activity of chromatin for transcription by homologous RNA polymerase II is reduced following in vivo treatment with DMN, the corresponding activity of purified DNA was subsequently examined. Three separate aspects of DNA transcription were examined: (a) the number of initiation sites per unit mass of DNA; (b) the rate of formation of initiation complexes; and (c) the rate of RNA chain elongation. Chart 4 represents the results of the titration of a constant amount of RNA polymerase II with increasing amounts of DNA obtained from control and DMN-treated animals. It is evident that the equivalence point (arrow) is the same for the 2 kinds of DNA, showing that no change in the number of initiation sites per unit mass of DNA occurs. Furthermore, the rate of formation of the initiation complexes is also unaltered by treatment with the carcinogen, formation being essentially complete within 5 min (Chart 5). Finally, the rate of elongation of the nascent RNA chains was measured after allowing sufficient time for full formation of the initiation complexes. As indicated in Chart 6, the kinetics of RNA chain elongation is not significantly affected following treatment with DMN.

Table 5

<table>
<thead>
<tr>
<th>DNA synthesis (cpm/min)</th>
<th>Chromatin from Control</th>
<th>Chromatin from DMN</th>
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<tr>
<td></td>
<td>Control RNA (polymerase II)</td>
<td>DMN RNA (polymerase II)</td>
</tr>
<tr>
<td>Control</td>
<td>7210 ± 390</td>
<td>7080 ± 350</td>
</tr>
<tr>
<td>DMN-treated</td>
<td>1600 ± 100</td>
<td>1790 ± 100</td>
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<td>% of inhibition</td>
<td>75 ± 3</td>
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* The initial rate of the reaction (used for the calculation of the results) was linearly related to chromatin concentration in this region.

* Average ± S.E. of at least 2 determinations.

Table 6

| Distribution of RNA polymerases between free and chromatin-bound forms |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                             | Free                        | Chromatin-bound             |
| RNA polymerase from         | RNA polymerase I            | RNA polymerase II           | RNA polymerase I            | RNA polymerase II           |
| Control                     | 4850 ± 450                  | 2990 ± 300                  | 940 ± 100                   | 3170 ± 290                  |
| DMN-treated                 | 5030 ± 460                  | 2870 ± 310                  | 710 ± 80                    | 3022 ± 290                  |
| % of inhibition             | NS                          | NS                          | NS                          | NS                          |

* Average ± S.E. of at least 2 determinations.

NS, not significant at the 95% level.

Chart 4: Titration of RNA polymerase II with purified DNA. A constant amount of RNA polymerase II, obtained from the livers of control animals and partially purified by DEAE-Sephadex column chromatography, was used to transcribe increasing amounts of DNA isolated from the livers of control or DMN-treated animals. Transcription was carried out for 15 min under conditions which permitted initiation but not elongation of RNA chains to occur (see "Materials and Methods"). O, DNA from control animals; x, DNA from animals treated with DMN (40 mg/kg) for 4 hr. Arrow, point of saturation of the RNA polymerase by DNA.

more, the rate of formation of the initiation complexes is also unaltered by treatment with the carcinogen, formation being essentially complete within 5 min (Chart 5). Finally, the rate of elongation of the nascent RNA chains was measured after allowing sufficient time for full formation of the initiation complexes. As indicated in Chart 6, the kinetics of RNA chain elongation is not significantly affected following treatment with DMN.

Appearance of Poly(A)-containing RNA and rRNA in the Cytoplasm. The effect of DMN on the rate of appearance of poly(A)-containing RNA and rRNA in the cytoplasm was examined by measuring the specific activities of these species at different times after injection of [3H]orotic acid. As can be seen from the data of Chart 7, the rate of appearance of poly(A)-containing RNA is reduced by approximately 50% following treatment with DMN at all time points examined. It is striking that this inhibition is significantly smaller than the corresponding inhibition of hnRNA synthesis which is caused by the same treatment with the carcinogen (about 80%). For the possible significance of this difference, see the "Discussion."
Effects of DMN on Mouse Liver RNA Synthesis

Chart 5. Kinetics of formation of initiation complexes between RNA polymerase II and DNA. Reaction mixtures were incubated for varying lengths of time under conditions permitting only formation of initiation complexes (see "Materials and Methods"), after which elongation was allowed to occur for 15 min. O, DNA from control animals; •, DNA from animals treated with DMN (40 mg/kg) for 4 hr.

Charts. Kinetics of formation of initiation complexes between RNA polymerase II and DNA. Reaction mixtures were incubated for varying lengths of time under conditions permitting only formation of the initiation complexes (see "Materials and Methods"), after which elongation was allowed to occur for 15 min. O, DNA from control animals; •, DNA from animals treated with DMN (40 mg/kg) for 4 hr.

Although, as mentioned earlier, DMN treatment at 40 mg/kg for 4 hr inhibits the synthesis of nucleolar RNA by about 30%, the corresponding effect on the appearance of mature rRNA in the cytoplasm is much more extensive. As can be seen from Chart 8, DMN causes a dramatic reduction in the amounts of the 3 rRNA species which appear in the cytoplasm 6 hr after injection of [3H]orotic acid, reaching 81, 83, and 87% for 4S, 18S, and 28S RNA, respectively.

DISCUSSION

Administration of a single dose of DMN to C57BL mice causes a rapid inhibition of the synthesis of nuclear hnRNA. The time course of the development of this effect (maximal inhibition is reached after 2 hr) (Chart 2) suggests that it is mediated by metabolism of the nitrosamine and, consequently, methylation of the cell constituents. It is noted that metabolism of DMN in the mouse takes place at a rate of approximately 20 mg/kg/hr (14) and results in the formation of methylating electrophilic species. The effects that we have examined refer to the ability of the cellular metabolic machinery, following its methylation by DMN, to synthesize and process RNA.

The observed inhibition of RNA synthesis is in general agreement with the results reported previously by Hultin et al. (10). Although these workers made no attempt to analyze separately and in detail the effects of DMN on the metabolism of the various types of RNA, they reported preferential inhibition of the Mn²⁺:(NH₄)₂SO₄-stimulated (i.e., RNA polymerase II-catalyzed) RNA-synthetic activity relative to the Mg²⁺-stimulated (i.e., RNA
differences between the mouse and the rat as far as RNA synthesis is concerned. Thus, implying the existence of species-specific recognition sites on RNA polymerase I molecules.

Methylation of DNA is known to reduce its activity as a template for transcription in a number of systems (8, 15). Ramstein et al. (28) have presented evidence that DNA methylation reduces the rate of RNA chain elongation during transcription. Using the DNA obtained from the livers of our DMN-treated mice 4 hr after a high dose (40 mg/kg) of the carcinogen, we have found no change in its behavior as a template for \textit{in vitro} RNA synthesis by homologous partially purified RNA polymerase II (Charts 4 to 6). The number and the rate of formation of the initiation complexes as well as the rate of RNA chain elongation were not different from those observed with control animal DNA. These results are in sharp contrast with the greatly reduced template activity of chromatin obtained from the same animals. It is possible that this difference may be due to the limitations of \textit{in vitro} transcription of DNA which results in extensive nonspecific initiation. However, it is interesting to refer to the levels of DNA methylation which have been found to be necessary to affect transcription significantly according to the earlier reports mentioned above. Thus, utilizing conditions which minimized nonspecific template DNA damage (strand breakage and depurination), Chen (6) and Marnat-Bratley (15) found significant inhibition of transcription only at levels of DNA methylation over 0.1\% of the nucleotides. A similar requirement for relatively extensive DNA methylation was found by Ramstein et al. (28). In contrast, the level of methylation of the DNA used in our experiments was never over 0.016\% of the nucleotides,4 a level expected to be inadequate to produce inhibition of \textit{in vitro} RNA synthesis even on otherwise intact templates. Consequently, it can be concluded that either DNA methylation affects transcription under the highly restrictive conditions which prevail \textit{in vivo} much more effectively than in relatively crude \textit{in vitro} systems or the carcinogen-induced methylation of macromolecules (including DNA and chromosomal proteins) results in alterations of chromatin structure which interfere with the operation of the transcription machinery. We envisage examining these possibilities further by studying the expression of specific genes.

In contrast to the extensive inhibition of hnRNA caused by DMN, nucleolar RNA synthesis is reduced by a relatively small degree. The mechanism of this effect was not investigated in detail. However, as with RNA polymerase II, both the activity and the \textit{in vivo} binding to chromatin of RNA polymerase I were not significantly affected. The template activity of nucleolar chromatin (which constitutes only a very small fraction of total chromatin) was not measured. The smaller degree of inhibition of nucleolar RNA synthesis (relative to hnRNA synthesis) is unlikely to be the result of differences in the extents of DNA methylation in the nucleus and the nucleolus, as it is known that in rat liver formation of O\textsuperscript{6}-methylguanine (one of the products of DNA methylation by DMN) is the same in the 2 classes of DNA (12).

DMN is known to cause a rapid and extensive inhibition of protein synthesis in the liver of rats (36) and mice (21–23). Thus, within 2 hr of the injection of DMN (37.5 mg/kg), protein synthesis in the livers of C57BL mice is almost completely blocked (23). The relevance of this finding to the present work lies in the fact that synthesis and maturation of nucleolar RNA are known to be coupled to protein synthesis. A number of studies have indicated that inhibition of protein synthesis is followed by a reduction in the rate of synthesis of nucleolar RNA, possibly due to feedback

\footnote{A. Hadjiof, personal communication.}

\footnote{Unpublished observations.}
that the observed effect of DMN on nucleolar RNA synthesis was secondary to the inhibition of protein synthesis. This is made more likely by our finding of a dramatic inhibition of the appearance in the cytoplasm of mature rRNA, a process expected to reflect most directly any changes in the rate of processing and transport of nucleolar RNA (Chart 8). One should also mention the possible direct control of nucleolar RNA synthesis and processing by hnRNA species, suggested by the experiments of Schmidt and Sekeris (30).

Following its synthesis in the nucleus, hnRNA undergoes processing and transport to the cytoplasm where it appears as a mRNA. Administration of DMN at a dose which inhibited the synthesis of nuclear hnRNA by 80% also resulted in reduction of the rate of appearance of poly(A)-containing mRNA in the cytoplasm, although only by 50% (Chart 7). This result, which confirms and extends a previous observation of Hultin et al. (10), suggests that the hnRNA synthesized following administration of DMN is processed and transported to the cytoplasm either faster or less specifically than normal. Both possibilities are compatible with available data, because it is well established that, following animal treatment with various carcinogens, including DMN, new RNA sequences can be detected in the cytoplasm (4, 33), indicating altered specificity of RNA processing and transport. Furthermore, the energy requirements for nucleocytoplasmic RNA transport appear to be partially abolished, at least as studied in vitro (31, 34), a factor which could result in altered kinetics of RNA transport in vivo.

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