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Inhibition of RNA Polymerase III by *N*-Hydroxy-2-Acetylaminofluorene (40902)

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Abstract. Parenteral administration of the potent hepatocarcinogen *N*-hydroxy-2-acetylaminofluorene to rats results in a substantial inhibition of hepatic RNA polymerase III(C), the enzyme responsible for the synthesis of tRNA and 5 S RNA. The minor component of RNA polymerase III extractable from nuclei shows a 45–65% reduction in activity while the major component of the enzyme extractable from cytoplasm shows a 40–65% inhibition. This reduction of RNA polymerase III activity is sufficient to account for the inhibition of rRNA synthesis produced by administration of the carcinogen *in vivo*.

Parenteral administration of the hepatocarcinogen *N*-2-acetylaminofluorene (AAF) or its proximate carcinogen *N*-hydroxy-2-acetylaminofluorene (H-OH AAF) to male rats or mice results in profound inhibition of hepatic RNA synthesis within several hours (1–3). All classes of RNA are affected. While mechanisms responsible for this inhibition of transcription remain controversial, we (4) and others (5, 6) have shown that at least part of the depression of rRNA synthesis can be accounted for by an inhibition of RNA polymerase I(A) and all of the inhibition of HnRNA synthesis can be explained by a reduction in RNA polymerase II(B) activity (5, 6). However, the inhibition of tRNA synthesis, which is at least as great as that of rRNA synthesis (1), has remained totally unexplained. We now report the results of our studies on the effects of N-OH AAF on hepatic RNA polymerase III(C), the enzyme responsible for synthesis of tRNA as well as 5 S RNA (7, 8). Since the majority but not all of RNA polymerase III activity is found associated with the cytoplasm when standard extraction procedures are employed (9, 10), it was necessary for us to examine both nuclear and cytoplasmic components of the enzyme. Our findings indicate that total RNA polymerase III activity is inhibited to a degree sufficient to account for the reported inhibition of tRNA synthesis by the carcinogen.

Materials and methods. N-OH AAF was obtained from the Aldrich Chemical Co., Milwaukee, Wisconsin. The absence of detectible impurities in this carcinogen was determined by thin layer chromatography (11). The melting point (147°C, Ref. (12)) and the infrared spectra of the compound confirmed its identity as N-OH AAF.

Male Sprague–Dawley rats (150–180 g) were housed under a 12-hr day–night lighting schedule at 25°C. They were maintained on Purina Lab Chow and fasted 12–16 hr before experiments. N-OH AAF (30 mg/kg) was injected intraperitoneally in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml. Control animals received DMSO alone. The animals were sacrificed by decapitation 2 hr later, a time when RNA synthesis is maximally depressed *in vivo* (1–3).

RNA polymerase. Nuclear and cytoplasmic extracts were prepared from the livers of control and carcinogen-treated rats by the method of Seifart *et al.* (9). Extraction of cytoplasmic RNA polymerase (almost entirely RNA polymerase III) and further purification of the enzyme by high speed centrifugation and DEAE cellulose chromatography were performed as described by the same authors (9). Quantitative solubilization of the nuclear enzymes (a mixture of RNA polymerases I, II, and III) was done by sonication of the nuclear lysate in high salt followed by high speed centrifugation as described by Roeder and Rutter (13). The solubilized enzymes were then fractionated by DEAE–Sephadex chromatography as previously described (4). The respective identities of RNA poly-

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merases I, II, and III were determined by their different sensitivities to the fungal toxin α-amanitin. RNA polymerase was assayed as previously described (14) using native calf thymus DNA as template, [^{3}H]UTP as labeled precursor, and 3.3 m*M* $MnSO_4$ as divalent cation. Protein concentrations were determined by the coomassie blue method (15) or by absorption at 280 nm.

Results. Inhibition of hepatic RNA polymerase III activity in crude nuclear and cytoplasmic extracts. The yields of RNA polymerases in crude nuclear and cytoplasmic extracts prepared from the livers of DMSO-treated (control) and N-OH-AAF-treated rats are shown in Table I. By comparing the total activity with that obtained in the presence of 1 μg/ml α-amanitin (which totally inhibits RNA polymerase II) and in the presence of 200 μg/ml α-amanitin (which totally inhibits RNA polymerase II and inhibits RNA polymerase III about 70%, Ref. (9)), one can get a good estimate of the levels of RNA polymerases I, II, and III in crude extracts.

Under the assay conditions employed, which favor RNA polymerase III, about one-half of the total RNA polymerase activity in control rats was found in the nuclear extracts and one-half in the cytoplasmic extracts. Nuclear activity consisted of large amounts of RNA polymerases I and II and a small amount of polymerase III. Cytoplasmic activity, on the other hand, consisted mainly of RNA polymerase III. (Since inhibition of RNA polymerase III by 200 μg/ml of α-amanitin is only about 70% (9), most of the α-amanitin resistant fraction in the cytoplasm is actually polymerase III while that in the nucleus is polymerase I).

In N-OH-AAF-treated rats, the distributions of the various hepatic RNA polymerases remained much the same with polymerases I and II being found principally in the nucleus and polymerase III in the cytoplasm. However, the levels of all three RNA polymerases were markedly lower in the N-OH-AAF-treated animals relative to those in controls. RNA polymerase III activity, in particular, was re-

TABLE I. RNA POLYMERASE ACTIVITY IN NUCLEAR AND CYTOPLASMIC EXTRACTS OF LIVER FROM DMSO AND N-OH-AAF-TREATED RATS

Cell extract	Treatment	RNA polymerase activity (pmole/g liver)[a]				
		α-Amanitin				
		None (A)	1 μg/ml (B)	200 μg/ml (C) (≃ polymerase I)[b]	Polymerase II (A−B)	Polymerase III (B−C)
Nuclear	DMSO	943 ± 32[c]	317 ± 16	223 ± 9	626 ± 33	94 ± 18
	N-OH AAF	499 ± 36[c] (53)[d]	168 ± 20 (53)	116 ± 40 (52)	331 ± 25 (53)	52 ± 39 (55)
Cytoplasmic	DMSO	936 ± 67	958 ± 36	300 ± 54	0	658 ± 54
	N-OH AAF	641 ± 6 (69)	611 ± 36 (64)	235 ± 39 (78)	30 ± 33 (−)	376 ± 55 (57)

[a] Nuclear and cytoplasmic extracts were prepared from five control and five N-OH-AAF-treated rats and assayed individually for RNA polymerase activity under the several stated conditions. Yields of DNA (mg/g liver; mean ± SEM) were: controls, 2.34 ± 0.11; N-OH AAF treated, 2.25 ± 0.05). Yields of protein in crude extracts (mg/g liver; mean ± SEM) were: control nuclear, 5.1 ± 0.6; N-OH AAF nuclear, 5.3 ± 0.5; control cytoplasmic, 124.0 ± 7.0; N-OH AAF cytoplasmic, 122.8 ± 3.2.

[b] Fraction C contains polymerase I plus 30% of any polymerase III present in the extract.

[c] Mean ± SEM.

[d] Values in parentheses = percentage of control.

duced by about 50% in nuclear extracts and 40–45% in cytoplasmic extracts. Comparable inhibitions of enzyme activity were observed whether results were expressed in terms of protein, DNA, or weight of liver.

Analysis of chromatographically separated RNA polymerases. Because of the existence of multiple species of enzyme in crude extracts it was necessary to confirm the inhibition of RNA polymerase III by comparing the activities of chromatographically separated enzymes from control and N-OH-AAF-treated animals. DEAE–Sephadex chromatography of total nuclear RNA polymerase (Fig. 1) consistently revealed inhibition of all three classes of RNA polymerase in carcinogen-treated animals relative to controls. (The various polymerases were differentiated by both chromatographic elution profile and α-amanitin sensitivity. Although not indicated in Fig. 1, combined Peak I fractions from both panels A and B were completely resistant to 200 μg/ml α-amanitin.) In confirmation of previous reports (4–6), RNA polymerase II activity was inhibited to the greatest extent (60–70%), while RNA polymerase I was inhibited to a lesser degree (35–45%). Nuclear RNA polymerase III, a minor fraction of total nuclear RNA polymerase, was inhibited by 45–65% in several experiments.

DEAE cellulose chromatography of cytoplasmic RNA polymerase (Fig. 2) revealed, in confirmation of the results of Seifart and Benecke (10), that almost all of the cytoplasmic RNA polymerase in control rat liver was RNA polymerase III, as defined by its chromatographic behavior and α-amanitin sensitivity. In some, but not all experiments, a small amount of leakage of RNA polymerase I into the cytoplasm was observed in N-OH-AAF-treated animals. In all cases the carcinogen-treated rats showed a substantial (40–65%) inhibition of cytoplasmic RNA polymerase III.

Attempts to restore the activity of peak column fractions of RNA polymerase III activity from N-OH-AAF-treated rats to control levels by the addition of column pass-through material derived from control or N-OH-AAF-treated animals were unsuccessful. Therefore, these experiments

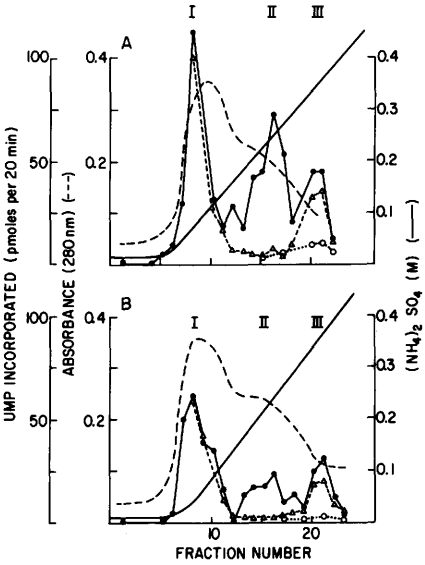

FIG. 1. DEAE–Sephadex column chromatography of nuclear RNA polymerases from control animals (panel A) and N-OH-AAF-treated animals (panel B). Three 180-g rats were injected intraperitoneally with N-OH AAF in dimethyl sulfoxide and three rats received solvent alone. Two hours after carcinogen administration, the animals were sacrificed and nuclear RNA polymerase was prepared as described in the text, from control and carcinogen-treated animals. Equal amounts (150 mg protein) of nuclear extract from control and AAF-injected animals were applied to identical 2 × 10-cm DEAE–Sephadex columns. Elution was performed using an $(NH_4)_2SO_4$ gradient. Aliquots (0.2 ml) of column fractions were assayed for polymerase activity using native calf thymus DNA as template and [^{3}H]UTP (20 cpm/pmole) as the labeled nucleoside triphosphate. "I," "II," and "III" refer to the three major classes of RNA polymerase, respectively. Salt concentrations were determined by conductivity measurements. RNA polymerase activity: in absence of α-amanitin (●——●), in presence of 1 μg/ml α-amanitin (△– –△), and in presence of 200 μg/ml α-amanitin (○···○).

provided no evidence that N-OH AAF was acting on a polymerase-associated factor, rather than on the enzyme itself.

Discussion. From these data it is evident that a single intraperitoneal injection of N-OH AAF rapidly inhibits hepatic RNA polymerase III activity. Both the small quantity of RNA polymerase III activity obtainable from nuclei and the major fraction of the enzyme found in the cytoplasm

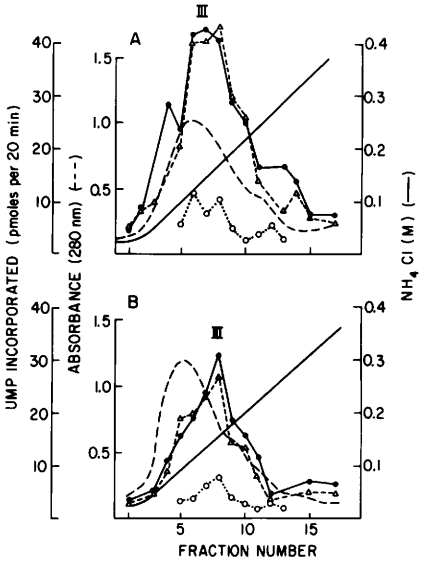

FIG. 2. DEAE-cellulose column chromatography of cytoplasmic RNA polymerase from control animals (panel A) and N-OH-AAF-treated animals (panel B). Cytoplasmic fractions from control and N-OH-AAF-treated animals described in Fig. 1 were processed as described by Seifart *et al.* (9) and equivalent 25-ml aliquots chromatographed on identical 5 × 8-cm DEAE-cellulose columns using an NH_4Cl gradient. The protein eluting with pass through fractions is not shown. Aliquots (0.2 ml) were assayed for RNA polymerase activity. RNA polymerase activity: in absence of α-amanitin (●——●), in presence of 1 μg/ml α-amanitin (△- -△), and in presence of 200 μg/ml α-amanitin (○·····○).

are affected. Since exogenous DNA was used as template in all assays, the observed reduction in RNA polymerase levels in livers of carcinogen-treated animals is not due to template alterations. Furthermore, the demonstration of reduced polymerase activity in both crude extracts and column-fractionated enzymes and the negative results of the mixing experiments argue in favor of inhibition of RNA polymerase III itself as opposed to inactivation of an easily dissociated "stimulatory" factor. The experiments do not differentiate between reduced numbers of enzyme molecules and reduced activity of individual enzyme molecules.

These experiments extend the previously known ability of N-OH AAF to inhibit RNA polymerases to include RNA polymerase III. The reduction in hepatic RNA polymerase III activity appears to be sufficient to account for the 40–50% inhibition of tRNA synthesis reported to occur (1) after parenteral administration of this carcinogen. Qualitative alterations in hepatic tRNA function have been described (16, 17) in N-OH-AAF-treated rats. Whether such changes are a reflection of altered RNA polymerase III activity or of posttranscriptional alterations of the tRNA molecules will require further investigation.

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