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Modification of Hepatic Ribonucleic Acid Polymerase Activities by *N*-Hydroxy-2-acetylaminofluorene and *N*-Acetoxy-2-acetylaminofluorene

ROBERT I. GLAZER[1]

Department of Pharmacology, Emory University, Atlanta, Georgia 30322

LARRY E. GLASS AND FREDERIC M. MENGER

Department of Chemistry, Emory University, Atlanta, Georgia 30322

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SUMMARY

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Treatment of partially hepatectomized rats with the hepatocarcinogen *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-AAF) inhibited the activities of nucleolar and nucleoplasmic RNA polymerase in hepatic nuclei. DEAE-Sephadex chromatography of nuclear RNA polymerases obtained from partially hepatectomized rats treated with *N*-hydroxy-AAF revealed stimulation in the activity of RNA polymerase I (nucleolar) and inhibition and an altered activity profile of RNA polymerase II (nucleoplasmic). No alterations were observed in the template activity of rat liver DNA from similarly treated animals. Assessment of the template activities of hepatic DNA after reaction with *N*-acetoxy-2-acetylaminofluorene (*N*-acetoxy-AAF) at 22° indicated that RNA polymerase II activity was affected to a greater degree than that of RNA polymerase I or *Escherichia coli* RNA polymerase, particularly at lower concentrations of carcinogens. In contrast, hepatic DNA that had reacted with *N*-acetoxy-AAF at 37° resulted in a 20–30-fold reduction in the concentration of activated carcinogen necessary to effect a 50% reduction in either rat liver or *E. coli* RNA polymerase activities. Whereas inhibition of nucleoplasmic RNA polymerase can account for the impairment in synthesis of extranucleolar species of RNA, it appears that inhibition of the synthesis of ribosomal RNA is not a direct result of reduction in nucleolar RNA polymerase or DNA template activity.

INTRODUCTION

Recent studies of the mechanism of action of *N*-hydroxy-2-acetylaminofluorene on liver RNA polymerase activities have suggested that a direct inhibitory effect upon the enzyme (1) or an altered transcriptional capacity of chromatin (2) may account for the inhibitory action of this

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proximate carcinogen upon RNA synthesis (3–6). Assessment of nucleolar and nucleoplasmic RNA polymerase activities in liver nuclei following treatment with *N*-hydroxy-AAF[2] has indicated an incongruity, in that preferential inhibition of nucleolar RNA polymerase (2) and equal inhibition of both nuclear enzymes (4) have been reported. Although these discrepancies may be dose-related (1), greater inhibition of the nucleoplasmic RNA polymerase compared to the nucleolar enzyme (1) was noted at a dose of *N*-hydroxy-AAF comparable to that used in the study by Grunberger *et al.* (2). An additional factor inherent in these investigations is the use of exogenous bacterial RNA polymerase (1, 7, 8) or deoxypolynucleotides (2) to measure whether damage or lack of damage has occurred to the enzyme molecule or chromatin, respectively.

To study this problem further, we have examined the template activity of DNA and the activities of crude and partially purified RNA polymerases from hepatic nuclei isolated from partially hepatectomized rats treated with *N*-hydroxy-AAF. In addition, a comparison was made of the relative sensitivities of RNA polymerases of rat liver and *Escherichia coli* for their ability to discriminate altered DNA template activity induced *in vitro* by *N*-acetoxy-AAF.

MATERIALS AND METHODS

Materials. [6-^{14}C]orotic acid (55 Ci/mmole), L-[*methyl*-^{3}H]methionine (4 Ci/mmole), [5,6-^{3}H]uridine 5′-triphosphate (37 Ci/mmole), [2-^{14}C]uridine 5′-triphosphate (50 mCi/mmole), and *S*-adenosyl-L-[*methyl*-^{3}H]methionine (5 Ci/mmole) were purchased from New England Nuclear Corporation. Poly(dA-T), UTP, GTP, CTP, ATP, and *E. coli* strain K-12 RNA polymerase were obtained from Sigma Chemical Company. *N*-Hydroxy-AAF, m.p. 148–149° (9), and *N*-acetoxy-AAF, m.p. 109.5–111° (10), were synthesized by published procedures.

[2] The abbreviations used are: *N*-hydroxy-AAF, *N*-hydroxy-2-acetylaminofluorene; *N*-acetoxy-AAF, *N*-acetoxy-2-acetylaminofluorene.

Treatment of animals. Male Sprague-Dawley rats (ARS/Sprague-Dawley) weighing 150–175 g were used in all experiments. Animals were maintained two per cage, containing corncob bedding, with alternating 12-hr periods of darkness and light.

Partial hepatectomies were performed under ether anesthesia between 1 and 2 p.m., according to the method of Higgins and Anderson (11). All animals were fasted for 20 hr and were allowed access to water ad libitum.

N-Hydroxy-AAF was dissolved in propylene glycol (8 mg/ml) and injected intraperitoneally 18 hr after partial hepatectomy at a dose of 20 mg/kg; control animals received an equivalent volume of propylene glycol.

Preparation of RNA polymerase. Rats treated with propylene glycol or *N*-hydroxy-AAF were killed 20 hr after partial hepatectomy, and liver nuclei were prepared by the method of Higashinakagawa *et al.* (12). RNA polymerases I and II were separated on DEAE-Sephadex according to Roeder and Rutter (13). Enzyme activity was assayed for 20 min at 37° in a reaction mixture (final volume, 0.25 ml) containing 20 μg of native calf thymus DNA (Sigma, type I), 50 mM Tris-HCl (pH 7.9), 1.6 mM $MnCl_2$, 1.6 mM 2-mercaptoethanol, 0.1 mM UTP, 0.6 mM GTP, 0.6 mM CTP, 0.6 mM ATP, and 1 μCi of [5,6-^{3}H]UTP. Nucleolar (Mg^{++}-dependent) and nucleoplasmic (Mn^{++}- and $(NH_4)_2SO_4$-dependent) RNA polymerase activities in isolated liver nuclei were assayed using 1 μCi of [5,6-^{3}H]UTP as radioactive precursor as described by Tata *et al.* (14).

Preparation of DNA. DNA was extracted from the nuclei of control or *N*-hydroxy-AAF-treated, partially hepatectomized rats by the sodium dodecyl sulfate extraction procedure of Okuhara (15). A water-saturated phenol–cresol mixture (1:7:2, v/v) containing 0.1% 8-hydroxyquinoline was used as the deproteinizing agent. RNA was removed from the DNA preparation by incubation with 100 μg of RNase A for 1 hr at 37° in 0.15 M NaCl and 0.04 M EDTA. Samples were then re-extracted with the phenol-cresol mixture, and the DNA was precipitated from the aqueous phase with 2 volumes of ethanol at −20°. DNA prepara-

tions had an A_{260}:A_{230} ratio of 2.30 ± 0.05 and an A_{260}:A_{280} ratio of 1.84 ± 0.05.

Reaction of DNA with N-acetoxy-AAF. Incubation mixtures contained the following components in 1 ml: 50 mM Tris-HCl (pH 7.9), 15 mM NaCl, 1.5 mM sodium citrate, 30% (v/v) ethanol, 25 μg of DNA, and *N*-acetoxy-AAF as indicated. After incubation for 1 hr at either 22° or 37° as indicated, reaction mixtures were extracted three times with 2 ml of ether, and the DNA was precipitated with 2 volumes of ethanol at −20° as described by Levine *et al.* (16). The DNA was further washed twice with ethanol, dried, and dissolved directly in the assay mixture used to measure RNA polymerase activity. By this technique, control DNA not reacted with *N*-acetoxy-AAF gave template activity comparable to that of DNA not subjected to the above incubation and extraction procedures.

Methylation of ribosomal RNA. Partially hepatectomized rats were treated with *N*-hydroxy-AAF as described, and pulsed for 15 min with 1 mCi/kg of L-[*methyl*-^{3}H]methionine and 50 μCi/kg of [6-^{14}C]orotic acid diluted in 0.9% NaCl (17). Ribosomal precursor RNA was extracted from liver nuclei at pH 5.1 by the sodium dodecyl sulfate–phenol extraction method described previously (18). Measurement of the extent of methylation in nuclei *in vitro* was carried out in the same incubation mixture used for assaying nucleolar RNA polymerase (14), with *S*-adenosyl-L-[*methyl*-^{3}H]methionine as methyl donor and [2-^{14}C]UTP as precursor to RNA. Incorporation of radioactivity from *S*-adenosyl-L-[*methyl*-^{3}H]methionine into acid-insoluble product occurred only under the assay conditions used to measure nucleolar RNA polymerase activity, and not under the assay conditions for the nucleoplasmic enzyme.[3]

RESULTS

The effect of treatment with *N*-hydroxy-AAF upon nucleolar and nucleoplasmic RNA polymerase activities in hepatic nuclei indicated that approximately equivalent inhibition of the two enzyme activities was produced (Table 1), a finding in agreement with previous reports (1, 4). To determine to what extent, if any, methylation of ribosomal RNA was involved in the action of *N*-hydroxy-AAF, double-labeling experiments were carried out both *in vivo* and *in vitro* (Table 2). Inhibition of methylation occurred in proportion to the impairment of RNA synthesis, suggesting that post-transcriptional methylation was reduced proportionally to the reduction in ribosomal precursor RNA. Furthermore, the similar extents of inhibition obtained for RNA polymerase activity and methylation are in close accord with the amount of impairment in the synthesis of nuclear and polyribosomal RNA previously attained (3). It should be noted that these effects were not associated with fluctuations in the pool size and specific radioactivity of UTP under the experimental conditions employed (3). Although pool sizes of methionine were not measured, the lack of changes in acid-soluble radioactivity[3] and the coincident findings of the measurement of methylation *in vitro* make this possibility unlikely.

Experiments were next initiated to de-

TABLE 1

Effect of N-hydroxy-AAF on nuclear RNA polymerase activities from partially hepatectomized male rats

Each animal received a single intraperitoneal injection of propylene glycol or *N*-hydroxy-AAF (20 mg/kg) 18 hr after partial hepatectomy. Animals were killed at 20 hr, and nuclear RNA polymerase activities were assayed as described under MATERIALS AND METHODS. Each value represents the mean ± standard error of determinations from five animals. Low ionic strength refers to the Mg^{++}-dependent RNA polymerase activity, and high ionic strength refers to the RNA polymerase activity assayed in the presence of Mn^{++} and $(NH_4)_2SO_4$ (14).

Treatment	UMP incorporation	
	Low ionic strength	High ionic strength
	pmoles/min/mg DNA	
Propylene glycol	109 ± 7 (100%)	105 ± 5 (100%)
N-Hydroxy-AAF	54 ± 6[a] (50%)	33 ± 7[a] (31%)

[a] Statistically significant difference ($p < 0.001$) vs. propylene glycol-treated controls.

[3] Unpublished observations.

termine the effect of carcinogen treatment upon the activity profile of nuclear RNA polymerases chromatographed on DEAE-Sephadex (Fig. 1). Liver nuclei obtained from control, partially hepatectomized rats contained two peaks of activity, corresponding to RNA polymerases I (nucleolar) and II (nucleoplasmic) (13, 19). Elution of enzyme activity from nuclei preparations obtained from animals treated with *N*-

TABLE 2

Effect of treatment with N-hydroxy-AAF on methylation of nuclear ribosomal RNA in vivo and in vitro

Animals were treated as described in Table 1 and under MATERIALS AND METHODS. Methylation and synthesis of rRNA were measured *in vivo* by incorporation of L-[*methyl*-^{3}H]methionine (1 mCi/kg) and [6-^{14}C]orotic acid (50 μCi/kg), respectively. Methylation and synthesis *in vitro* were determined by incorporation of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (5 μCi/nmole) and [2-^{14}C]UTP (0.3 μCi/20 nmoles), respectively, under the assay conditions employed for the measurement of Mg^{++}-dependent RNA polymerase in nuclei as described (14). Each value is the mean ± standard error of determinations from three animals.

Treatment	*In vivo*		*In vitro*	
	^{3}H	^{14}C	^{3}H	^{14}C
	cpm/A$_{260}$	*cpm/A*$_{260}$	*pmoles/min/mg DNA*	
Propylene glycol	3720 ± 410 (100%)	6220 ± 830 (100%)	2.6 ± 0.1 (100%)	81 ± 3 (100%)
N-Hydroxy-AAF	1300 ± 180[a] (35%)	2670 ± 460[a] (43%)	1.5 ± 0.2[a] (58%)	42 ± 6[a] (52%)

[a] Statistically significant difference ($p < 0.05$) compared with propylene glycol-treated controls.

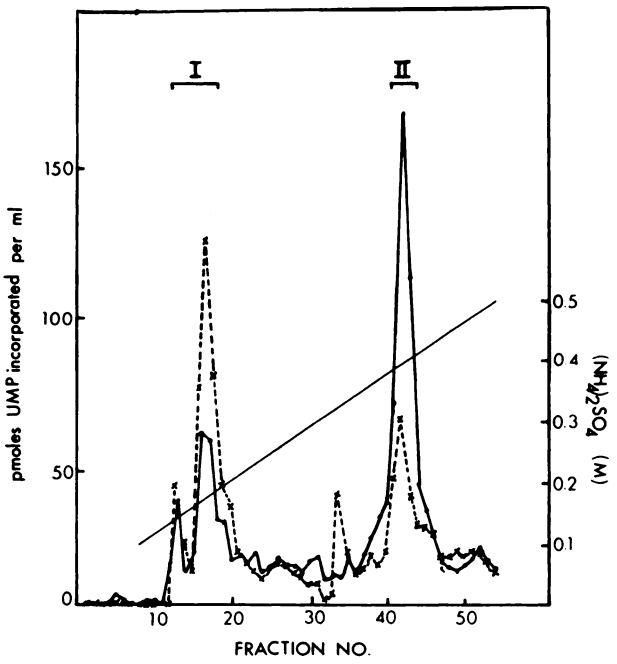

FIG. 1. *DEAE-Sephadex chromatography of nuclear rat liver RNA polymerases from partially hepatectomized rats following treatment with N-hydroxy-AAF*

Partially hepatectomized rats were treated with propylene glycol or *N*-hydroxy-AAF as described in Table 1. RNA polymerases were extracted from nuclei and chromatographed as described under MATERIALS AND METHODS. ●——●, propylene glycol treatment; x- - -x, *N*-hydroxy-AAF treatment. Specific activities (picomoles of UMP incorporated per milligram of protein per 20 min): propylene glycol treatment, peak I, 350; peak II, 15,800; *N*-hydroxy-AAF treatment, peak I, 650; peak II, 4500.

hydroxy-AAF revealed higher RNA polymerase I activity, with a concomitant reduction and somewhat altered activity profile of RNA polymerase II. These results were consistently obtained, with inhibition of RNA polymerase II from treated animals varying between 30% and 50% of control activity in four chromatographic runs. The activity of RNA polymerase I from carcinogen-exposed animals also differed with each chromatographic run, and ranged from 80% to 160% of control values for a similar number of experiments.

Interesting differences were noticed between the two rat liver enzymes in comparison to the bacterial RNA polymerase (Table 3). In agreement with the findings of other investigators (13, 19), RNA polymerase II showed a higher affinity for heat-denatured DNA than did RNA polymerase I; however, neither enzyme utilized poly(dA-T) to any significant extent. In contrast, not only did denatured DNA serve as a more active template than native DNA for *E. coli* RNA polymerase, but poly(dA-T) served as a more efficient template than denatured DNA.

Comparison of the template activity of DNA extracted from the livers of control and *N*-hydroxy-AAF-treated animals with rat liver and bacterial RNA polymerases is presented in Table 4. Although there was some depression in the template activity of DNA obtained from treated animals, there was neither a consistent nor a statistically significant difference in this effect on RNA synthesis.

To determine further whether the two mammalian enzymes would exhibit different binding affinities for a carcinogen-modified template, *N*-acetoxy-AAF was allowed to react *in vitro* with rat liver DNA and the extent of altered template capacity was monitored with rat liver and bacterial RNA polymerases (Fig. 2). Reactions with *N*-acetoxy-AAF carried out at 22° indicated that greater inhibition of enzyme activity was detected with RNA polymerase II, and that both liver enzymes were more sensitive monitors of template modification than the *E. coli* enzyme. DNA extracted from the livers of sham-operated or partially hepatectomized rats gave essentially the same results upon reaction with *N*-acetoxy-AAF.

Reaction of DNA with activated carcinogen at 37° resulted in equal inhibitory effects for the bacterial and rat liver RNA polymerases (Fig. 3), a result probably reflecting a greater extent of base substitution via arylamidation.

TABLE 3

Activity of rat liver and E. coli RNA polymerases with calf thymus DNA and poly(dA-T)

Liver RNA polymerases I and II were prepared from partially hepatectomized rats 20 hr after operation as described under MATERIALS AND METHODS. Assays were performed with 20 μg of template for 20 min at 37° and contained 15 μg of RNA polymerase I, 1 μg of RNA polymerase II, and 2 μg of *E. coli* RNA polymerase. Each value is the mean ± standard error of five separate preparations of DNA. Denatured DNA was prepared by heating native DNA for 10 min at 100°, followed by rapid cooling.

RNA polymerase	UMP incorporation with		
	Native DNA	Denatured DNA	Poly (dA-T)
	pmoles	*pmoles*	*pmoles*
Rat liver, I	6.40 ± 0.45	4.20 ± 0.45	0.10 ± 0.01
Rat liver, II	13.40 ± 1.25	27.90 ± 2.90	0.08 ± 0.01
E. coli	14.50 ± 1.00	30.50 ± 3.00	68.50 ± 5.00

DISCUSSION

Monitoring of alterations in DNA template activity induced via arylamidation with *N*-acetoxy-AAF has previously been examined only with exogenous bacterial RNA polymerases (1, 7, 8). The present study is the first to compare the characteristics of nucleolar RNA polymerase I and nucleoplasmic RNA polymerase II of regenerating rat liver after treatment with *N*-hydroxy-AAF, and their relative abilities to detect carcinogen-induced template modification *in vitro*. It may be concluded that RNA polymerase II is more sensitive than RNA polymerase I and *E. coli* RNA polymerase to the smaller degrees of template alkylation that occur by reaction of DNA with *N*-acetoxy-AAF at 22°. In contrast, the presumably greater base substitution occurring in DNA at 37° did not reveal any striking differences between the mammalian and bacterial enzymes. Despite these

TABLE 4

Template activity of DNA obtained from partially hepatectomized rats after treatment with N-hydroxy-AAF

Rat liver RNA polymerases I and II were prepared from partially hepatectomized rats treated with either propylene glycol or 20 mg/kg of *N*-hydroxy-AAF as described in Table 1. DNA was extracted from similarly treated animals. See MATERIALS AND METHODS for experimental details. Assay conditions were the same as described in the legend to Table 3. Each value is the mean ± standard error of duplicate assays of two separate enzyme preparations using six preparations of DNA.

Source and type of RNA polymerase	UMP incorporation	
	Control liver DNA	*N*-Hydroxy-AAF-treated liver DNA
	pmoles	*pmoles*
Control liver, I	5.60 ± 0.40 (100%)	5.60 ± 0.90 (100%)
Control liver, II	11.40 ± 0.95 (100%)	9.45 ± 2.00 (83%)
N-Hydroxy-AAF-treated liver, I	9.00 ± 0.80 (100%)	7.80 ± 0.80 (87%)
N-Hydroxy-AAF-treated liver, II	4.50 ± 0.50 (100%)	4.15 ± 0.50 (92%)
E. coli	9.80 ± 0.70 (100%)	7.85 ± 1.30 (80%)

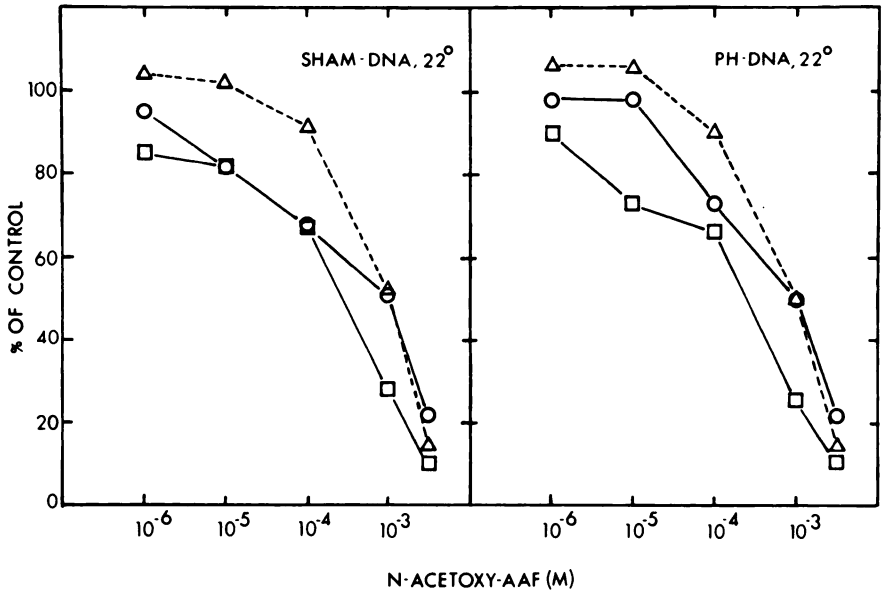

FIG. 2. *Inhibition of RNA polymerase activities by treatment of DNA from sham-operated or partially hepatectomized rats with N-acetoxy-AAF at 22°*

DNA extracted from sham-operated (sham-DNA) or partially hepatectomized (PH-DNA) rats was incubated with the indicated concentrations of *N*-acetoxy-AAF for 60 min at 22°. Following incubation residual *N*-acetoxy-AAF was extracted as described under MATERIALS AND METHODS, and the reacted DNA (25 μg) was assayed using rat liver RNA polymerase I (15 μg) (O——O), rat liver RNA polymerase II (1 μg) (□——□), or *E. coli* RNA polymerase (2 μg) (Δ- - -Δ). In the presence of unreacted DNA the amounts (picomoles) of UMP incorporated in 20 min at 37° were: rat liver RNA polymerase I, 5; rat liver RNA polymerase II, 10; *E. coli* RNA polymerase, 10.

similarities, it is important to note that characteristic differences do exist between these enzymes in their utilization of various templates. The bacterial RNA polymerase has a high affinity for denatured DNA, as does RNA polymerase II, but differs from both liver enzymes in its efficient utilization of the double-stranded copolymer, poly(dA-T). This appears to be an important point, since the latter tem-

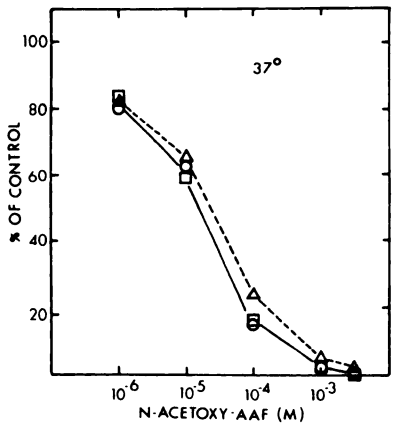

FIG. 3. *Inhibition of RNA polymerase activities by treatment of DNA from partially hepatectomized rats with N-acetoxy-AAF at 37°*

Reaction of DNA with *N*-acetoxy-AAF was performed as described in Fig. 2, except that incubation was performed at 37°. See the legend of Fig. 2 for the assay conditions for RNA polymerase activities. O——O, rat liver RNA polymerase I; □——□, rat liver RNA polymerase II; Δ- - -Δ, *E. coli* RNA polymerase.

plate has been used to assess whether endogenous template changes have occurred in assays of RNA polymerase activity in isolated nuclei following treatment of animals with *N*-hydroxy-AAF (2). It is evident from our experiments that the RNA polymerases of rat liver *cannot* utilize the polydeoxynucleotide to any great extent, casting doubt on the validity of using this template in crude systems which cannot distinguish between true RNA polymerase and copolymer-forming enzyme activities.

The extent of base substitution under conditions *in vitro* was not directly measured in the present study; however, the sigmoid concentration-response curves indicate that reactivity is concentration- and temperature-dependent. Troll *et al.* (7) estimated that 65% substitution occurs after incubation of DNA with 30 mM *N*-acetoxy-AAF for 1 hr at 37°, while Levine *et al.* (16) detected 4% substitution by 10 mM *N*-acetoxy-AAF under reaction conditions similar to those used in the present study. In contrast, bacterial DNA appears to be more susceptible to alkylation, with 26% base substitution occurring at 1 mM *N*-acetoxy-AAF (20). It has generally been found that for significant denaturation of DNA to occur by reaction with *N*-acetoxy-AAF, at least 10% of the guanine residues must be affected (16, 21). This conclusion has been based on the behavior of DNA on hydroxylapatite (16) and on thermal denaturation studies (20). Analysis of alkylated DNA by means of its buoyant density has shown that as little as 4% substitution can affect this parameter (16, 20). Therefore it appears from the present investigation, and from those of others (7, 8), that lesser degrees of modification of the template activity of DNA can be determined with RNA polymerase at reaction concentrations of *N*-acetoxy-AAF producing no overt denaturation of DNA as determined by other analytical techniques.

Maximal binding of *N*-hydroxy-AAF to liver DNA occurs within 1–2 hr after intraperitoneal administration (22, 23), and is temporally coincident with maximal inhibition of the synthesis of nuclear and cytoplasmic RNA (1, 3) and RNA polymerase activities (1, 2). However, the binding of *N*-hydroxy-AAF to DNA *in vivo* does not necessarily denote a causal relationship with its effects on RNA synthesis. Indeed, evidence obtained from experiments with *N*-hydroxy-AAF *in vivo* indicates that there is a low degree of base substitution (less than 0.1%) of DNA (24, 25). The present findings of a minimal effect by *N*-hydroxy-AAF *in vivo* on DNA template activity, as well as inhibition of nucleoplasmic RNA polymerase, suggest that nuclear proteins involved in the regulation of the synthesis of RNA may serve as important subcellular targets for this carcinogenic metabolite. Furthermore, the 3-fold lesser sensitivity of liver DNA polymerase to changes in priming activity of DNA induced by *N*-acetoxy-AAF (7) signifies that perturbation of RNA synthesis may be one of the most sensitive biosynthetic processes affected by this class of hepatocarcinogens.

In conclusion, it is apparent that the proximate carcinogen *N*-hydroxy-AAF exerts a potent inhibitory effect on nucleoplasmic RNA polymerase, the enzyme presumably involved in the synthesis of mes-

senger RNA, or at least a DNA-like RNA product (14, 26). A potential ultimate carcinogenic metabolite of *N*-hydroxy-AAF, *N*-acetoxy-AAF, altered the template activity of DNA *in vitro* and the subsequent synthesis of RNA, although no effect of this type was produced after treatment *in vivo* with *N*-hydroxy-AAF. These results suggest that there may be two mechanisms of action of *N*-hydroxy-AAF on RNA synthesis. One effect would encompass direct inhibition of nucleoplasmic RNA polymerase, resulting in cessation of synthesis of messenger RNA. The other action of *N*-hydroxy-AAF would pertain to inhibition of ribosomal RNA, where no overt effects by this carcinogen have been produced thus far on either the template function of DNA or the RNA polymerase molecule. Since other regulatory proteins might be acting in concert with the nucleolar enzyme in some regulatory manner as suggested by the recent work of Higashinakagawa *et al.* (27), the locus of action for the hepatocarcinogen may well be at this or some other chromosomal protein site.

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