The Effect of Modification of T7 DNA by the Carcinogen *N*-2-Acetylaminofluorene: Termination of Transcription in Vitro†

Robert L. Millette‡ and Louis M. Fink*

ABSTRACT: To study the effects of *N*-2-acetylaminofluorene (AAF) modification of DNA on transcription, purified DNA from bacteriophage T7 was modified in vitro to varying extent with AAF and transcribed by DNA-dependent RNA polymerase from *Escherichia coli*. The main effects of AAF modification on transcription are a marked inhibition of the rate and extent of RNA synthesis with relatively little effect on initiation except at very high AAF doses. Calibration of the percent modification with [^{14}C]AAF and analysis of the size of the RNA product by double isotope labeling and polyacrylamide gel electrophoresis support the following mechanism of transcription inhibition: most of the AAF residues bound to the coding strand of the DNA cause premature termination of transcription, at or near the site of modification, with release of RNA polymerase. This results in the production of shorter RNA chains with increasing amounts of bound carcinogen. The data are consistent with there being no reinitiation and/or synthesis of RNA distal to the AAF-modification site.

The model carcinogen *N*-acetoxy-2-acetylaminofluorene (N-AcO-AAF)[1] prepared by Miller et al. (1966) reacts with nucleic acids in vitro. The predominant reaction product is a covalent binding of the *N*-2-acetylaminofluorene (AAF) residue to the C-8 position of the guanine moieties (Kriek et al., 1967). This modification has been shown to result in alterations in the secondary structure of nucleic acids (Kapuler and Michelson, 1971; Fuchs and Daune, 1972; Levine et al., 1974). Physical studies with DNA indicate that covalent attachment of AAF produces localized regions of denaturation without strand scission and support a base displacement model in which the modified bases are shifted out of the double helix while the covalently bound carcinogen residue is inserted (Levine et al., 1974).

The carcinogen binding also affects the biological activity of nucleic acids. tRNAs containing one or two residues of AAF are impaired in their ability to be aminoacylated by tRNA aminoacyl synthetases (Fink et al., 1970; Weinstein et al., 1971; Pulkrabek et al., 1973). Synthetic oligo- and polyribonucleotides containing AAF modified guanosine show marked reduction in ribosome binding assays with no evidence of miscoding (Grunberger et al., 1970, 1971). Translation of AAF-modified polyribonucleotide templates in vitro is strongly reduced (Grunberger and Weinstein, 1971).

We have been particularly interested in analyzing the effects of carcinogen modification of DNA on transcription. Several investigators have shown that AAF or N-OH-AAF administered in vivo alters the RNA synthesizing capacity of isolated liver nuclei or purified liver DNA (Troll et al., 1968; Dawson, 1972; Grunberger et al., 1973). Nucleolar RNA synthesis seems to be most markedly affected (Grunberger et al., 1973). Moreover, DNA modified in vitro with AAF is highly impaired in its template activity toward bacterial RNA polymerase (Troll et al., 1968, 1969). Although these studies have established that AAF modification of DNA inhibits the incorporation of ribonucleotides, they have not elucidated what effect the bound carcinogen has on the nature of the RNA synthesized nor the mechanism by which transcription is blocked.

Therefore, to examine in more detail the effects of AAF modification of DNA on the transcription process, we have utilized an in vitro transcription system of DNA-dependent RNA polymerase from *Escherichia coli* and AAF-modified bacteriophage T7 DNA. T7 DNA was chosen as a substrate for modification because its in vitro transcription product is predominantly a single RNA species (2.2×10^6 daltons) from the early gene transcription region (Millette et al., 1970) and is easily characterized by its mobility in polyacrylamide gel electrophoresis. The present studies show that the predominant effect of AAF modification of DNA on transcription is the inhibition of the rate of RNA synthesis. Analysis of the RNA products demonstrates that this inhibition is due to the induction of premature chain termination with the resultant production of shorter RNA chains by RNA polymerase.

Materials and Methods

Materials. N-AcO-AAF and N-OH-AAF were generously supplied by Dr. James Miller of the University of Wisconsin. [9-^{14}C]-N-OH-AAF, 14.8 Ci/mol (International Chemical and Nuclear Corp.), was diluted with unlabeled N-OH-AAF and converted to N-AcO-AAF (0.740 Ci/mol) using the method described by Maher et al.

† From the Department of Pathology, University of Colorado Medical Center, Denver, Colorado 80220. *Received September 9, 1974.* Supported by grants from the U.S. Public Health Service, CA-15823 and CA-15109-01 (L.M.F.), The Milheim Foundation for Cancer Research (L.M.F.), the National Science Foundation, GB-23356 (R.L.M.), and U.S. Public Health Service Research Career Development Award, 5-K04 GM-19333 (R.L.M.). A preliminary report of this work was presented at the annual meeting of the American Association for Cancer Research, Houston, Texas, March 1974.

‡ Present address: University of Chicago, Committee on Virology, Chicago, Illinois 60637.

[1] Abbreviations used are: AAF, *N*-2-acetylaminofluorene; N-OH-AAF, *N*-hydroxy-*N*-2-acetylaminofluorene; N-AcO-AAF, *N*-acetoxy-*N*-2-acetylaminofluorene; AAF-DNA or AAF-T7 DNA designates DNA reacted with AAF and repurified as described in the Methods section.

(1968). For each experiment a fresh stock solution of N-AcO-AAF in 100% ethanol was prepared.

Bacteriophage T7 was prepared as described by Summers and Szybalski (1968). T7 DNA was prepared by extracting the phage three times with phenol saturated with NET (0.1 *M* NaCl–1 m*M* EDTA–10 m*M* Tris-acetate (pH 7.5)) containing 0.08% (w/v) 8-hydroxyquinoline and two times with chloroform–octanol (9:1) and finally dialyzed extensively vs. 10 m*M* Tris-acetate (pH 7.9)–1 m*M* EDTA. α-^{32}P-labeled ribonucleoside triphosphates were synthesized by a modification of Symon's method (1968) as described by J. Dahlberg (personal communication) and were used at a specific activity of 1.5×10^3 cpm/pmol.

[γ-^{32}P]ATP and [γ-^{32}P]GTP were synthesized by the method of Glynn and Chappell (1964) but were purified from the reaction mixture by ion exchange chromatography on a 2-ml column of QAE-Sephadex, A-25 (Pharmacia). They were eluted with 0.2 *N* HCl, neutralized with NH_4OH, concentrated by rotary evaporation on a Vortex mixer to about 0.2 ml, and the salt removed by gel filtration on a 20-ml Sephadex G-10 column in the presence of 5 m*M* Tris-acetate (pH 7.9)–0.1 m*M* EDTA (J. Dahlberg, personal communication). The γ-^{32}P-labeled nucleoside triphosphates were used at a specific activity of $1.59–1.86 \times 10^3$ cpm/pmol.

DNA-dependent RNA polymerase was prepared from *Escherichia coli* K12 (3/4 log phase, washed, from Grain Processing Corp.) and stored as previously described (Millette and Trotter, 1970). It had a specific activity of 3250 enzyme units/mg of protein.

Modification of T7 DNA with N-AcO-AAF. For preparation of AAF-T7 DNA with unlabeled N-AcO-AAF, a fresh stock solution of 16.4 m*M* N-AcO-AAF in 100% ethanol was prepared and from this serial 1:10 dilutions in ethanol were made. From stock solution of T7 DNA (525 μg/ml in 10 m*M* Tris-acetate (pH 7.9)), 0.2 ml (105 μg of DNA) was added to 0.1 ml of (a) 16.4 m*M* N-AcO-AAF, (b) 1.64 m*M* N-AcO-AAF, and (c) 0.164 m*M* N-AcO-AAF. One aliquot of nucleic acid was incubated under identical conditions but in the absence of N-AcO-AAF. The mixtures were incubated 3 hr at 37° and then a 0.1 volume of 4 *M* NaCl and 2 volumes of cold ethanol were added. The samples were placed at −20° overnight and then the precipitate was collected by centrifugation at 10,000*g* for 30 min at 0°. The supernatant was removed and the precipitate was washed with cold ethanol. The modified DNA was resuspended in 0.5 ml of 10 m*M* Tris-acetate (pH 7.9)–1 m*M* EDTA.

Absorbancy measurements of the DNA were made on both the Zeiss PMQII and Cary 14 spectrophotometers. Concentrations of DNA were determined by absorbancy at 260 nm using a conversion value of 1 mg/ml of DNA = 20 A_{260} units/ml. The concentration of DNA in AAF-modified samples was computed with correction for the contribution of AAF to the 260-nm absorbancy (Fink et al., 1970). Since the amount of carcinogen bound to DNA is approximately proportional to the ratio A_{305}/A_{260}, this ratio was determined in order to estimate the relative degree of modification of the DNA (Kriek et al., 1967; Fink et al., 1970).

For preparation of [^{14}C]AAF-T7 DNA, 0.1 ml of 1.9 m*M* [^{14}C]-N-AcO-AAF in ethanol was added to 0.2 ml (258 μg) of T7 DNA. The mixture was incubated and the DNA precipitated as described above. The DNA was resuspended in 10 m*M* Tris acetate (pH 7.9)–1 m*M* EDTA–0.4 *M* NaCl and reprecipitated with ethanol. The uv absorbancy was measured and an aliquot was hydrolyzed with DNase I (Worthington) (20 μg/ml, 30 min at 37°) and counted in Kinard's solution (Kinard, 1957) in a Beckman scintillation counter. The percent modification was determined by calculating the micromoles of [^{14}C]AAF bound/micromole of nucleotide.

RNA Synthesis Assay. RNA synthesis was carried out in 50–100-μl reaction volumes as previously described (Millette and Trotter, 1970) but with 0.13 *M* NH_4Cl, 30 m*M* $Mg(OAc)_2$, phage T7 DNA at 80 μg/ml, and 80 μg/ml of RNA polymerase. For kinetic measurements, 10-μl aliquots were taken at various times after initiating synthesis and pipetted into 1 ml of ice–water, and the nucleic acids were precipitated with an equal volume of 10% Cl_3CCOOH. The precipitates were collected on Whatman GF/C glass fiber filters, washed three times with ice-cold 5% Cl_3CCOOH and two times with ice-cold ethanol, and dried, and radioactivity was measured in a liquid scintillation spectrometer.

For double labeling experiments [^{3}H]UTP at 24 Ci/mol (Schwarz BioResearch) and [γ-^{32}P]ATP and [γ-^{32}P]GTP at $1.59–1.86 \times 10^3$ cpm/pmol were used as labeled substrates. RNA synthesis was assayed as above but in order to prevent retention of the unincorporated ^{32}P-labeled nucleoside triphosphates on the filters, 10 μmol of unlabeled ATP was included in the 1 ml of ice–water and the filters were washed 10 times with a total of about 50–70 ml of 5% Cl_3CCOOH and then with ethanol.

Polyacrylamide Gel Electrophoresis of RNA. For this analysis, 5–15-μl aliquots of the T7 DNA transcription mixtures were removed at 45 min after commencing RNA synthesis, diluted into 30 μl of 0.2% sodium dodecyl sulfate, 5 m*M* EDTA, and 5 m*M* Tris-HCl (pH 7.2), heated at 65° for 3 min, and chilled on ice; 10 μl of 50% w/v sucrose in 10 m*M* EDTA–10 m*M* Tris-acetate (pH 7.2) was added and the samples were layered onto 2.4% polyacrylamide gels for electrophoresis. The polyacrylamide gels (0.5 × 9 cm) containing 0.2% sodium dodecyl sulfate were prepared (unswelled) and run (90 min at 90 V) according to Bishop et al. (1967). The gels were frozen on Dry Ice and sliced into 1.5-mm sections. The slices were dried on filter paper strips which were then cut into sections, and radioactivity was determined by liquid scintillation spectroscopy in a toluene scintillator (4 g of Omnifluor, New England Nuclear Corp., per 1 l. of toluene). ^{32}P-labeled 4S, 18S, and 28S L-cell RNA gel markers were kindly provided by Dr. Tom Walker.

Calculations

(1) The Number Average Chain Length of in Vitro Synthesized T7 RNA. Since DNA-dependent RNA polymerase initiates RNA chains with either ATP or GTP, the number average RNA chain length synthesized may be approximated from the ratio of total picomoles of nucleotides incorporated into RNA divided by the picomoles of chains initiated. Thus, the number average RNA chain length, $\overline{CL_n}$, = 4 [(picomoles of [^{3}H]UMP incorporated)/(picomoles of [γ-^{32}P]ATP + GTP) incorporated].

(2) The number of AAF induced chain termination sites per 1000 nucleotides in the template DNA =

$$\left[\frac{\overline{CL_n},\ \text{control DNA}}{\overline{CL_n},\ \text{AAF-DNA}} - 1\right]\frac{1000}{6730}$$

where $\overline{CL_n}$ = number average chain length of RNA synthesized, control DNA = unmodified T7 DNA, AAF-DNA = modified T7 DNA, and 6730 = the number of nucleotides

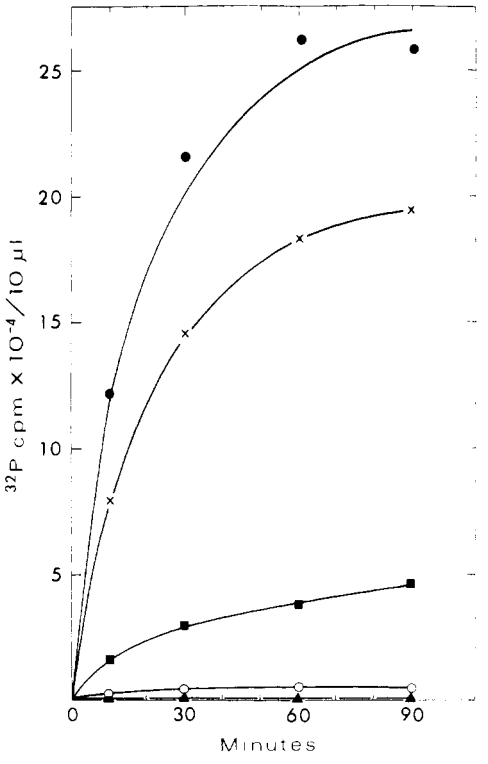

FIGURE 1: Kinetics of transcription of AAF-modified T7 DNA. RNA synthesis was assayed as described under Materials and Methods using 0.1-ml reaction volumes and the four α-^{32}P-labeled ribonucleoside triphosphates each at 45 cpm/pmol and 0.5 µmol/ml. Unmodified T7 DNA (●); T7 DNAs with increasing percent modification where A_{305}/A_{260} equals 0.0288 (X), 0.033 (■), 0.048 (○), and 0.168 (▲) (see Table I for data on these templates).

in the complete T7 early gene transcript.

(3) Molecular weight of the RNA in the polyacrylamide gels was determined from a linear plot of log molecular weight vs. gel slice numbers (electrophoretic mobility) using ^{32}P-labeled 18S and 28S L-cell RNAs and the intact 2.2 × 10^6 dalton in vitro synthesized T7 RNA (Millette et al., 1970) as standards.

(4) The relative number of RNA chains for each molecular weight class (gel slice number) was calculated from cpm_i/M_i, where cpm_i = radioactivity in slice i and M_i = molecular weight of RNA in slice i.

Results

Figure 1 shows the effects of increasing AAF modification of T7 DNA on the kinetics of in vitro transcription. Increasing modification by AAF causes a dramatic reduction in the rate of transcription and the amount of RNA synthesized at the various times. However, this alteration of the template does not appear to cause early cessation of transcription as synthesis continues on all active templates for at least 90 min. This indicates that the polymerase is not irreversibly trapped at the sites of modification. The high levels of AAF modification (A_{305}/A_{260} = 0.168) completely inhibit RNA synthesis.

To ascertain the nature of this inhibition, RNA synthesis was carried out with [^{3}H]UTP to monitor synthesis and [γ-^{32}P]ATP and GTP to measure RNA chain initiation (Figure 2). In contrast to the effects on synthesis, AAF modification has relatively little effect on RNA chain initiation. The intermediate AAF modification (A_{305}/A_{260} = 0.033) causes a slight stimulation while the high levels of modification (A_{305}/A_{260} = 0.048) cause some inhibition of initiation which is compatible with the small target size of the promoter region. The action of AAF modification of T7 DNA on the initial rates of RNA synthesis and initiation is tabulated from the average of two experiments in Table I.

Such data also allow calculations of the number average chain lengths of RNA synthesized on the various templates from the ratio of [^{3}H]UMP to γ-^{32}P-labeled nucleoside triphosphate incorporation (Table I). The RNA chain lengths decrease with increasing amounts of carcinogen bound to the template. Furthermore, this reduction in chain length parallels the observed decrease in the rate of RNA synthe-

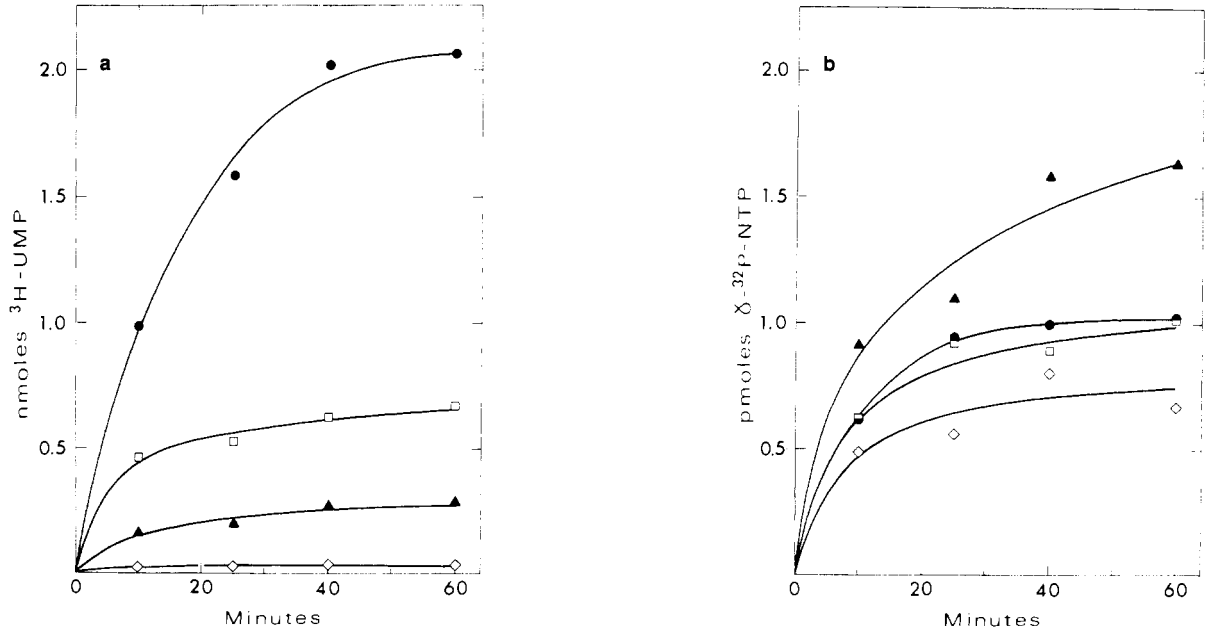

FIGURE 2: Kinetics of RNA synthesis and initiation with AAF-modified T7 DNA. RNA synthesis and initiation were assayed by the double labeling procedure described under Materials and Methods. (a) Kinetics of RNA synthesis as assayed by [^{3}H]UTP incorporation; (b) kinetics of RNA chain initiation as assayed by [γ-^{32}P]ATP and -GTP incorporation. Templates used were unmodified T7 DNA (●) and AAF-modified T7 DNA having A_{305}/A_{260} ratios of 0.0288 (□), 0.033 (▲), and 0.048 (◇) (see Table I).

Table I: Effects of AAF Modification on T7 DNA Transcription.

DNA	A	B	C	D
A_{305}/A_{260}[a]	0.0282	0.0288	0.0330	0.0483
Estimated % modification[b]	0	0.021	0.17	0.72
RNA synthesis rate[c]	1066	468	160	20.0
RNA initiation rate[d]	0.838	0.843	1.173	0.656
RNA chain length, nucleotides[e]	5554	2029	573	132
Chain termination sites/1000 nucleotides[f]	0	0.258	1.29	6.11

[a] A = control T7 DNA; B, C, and D = AAF-modified T7 DNAs. [b] Calibrated from [^{14}C]AAF-DNA (see Figure 3). [c] pmoles of [^{3}H]UMP incorporated per 10 min. [d] pmoles [γ-^{32}P]ATP and -GTP incorporated per 10 min. [e] Number average RNA chain length (see Methods). [f] See Methods.

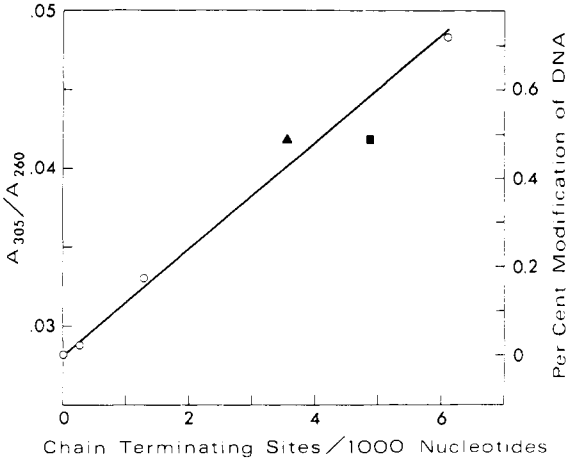

FIGURE 3: Correlation of A_{305}/A_{260} ratio of the DNA with percent AAF modification and polymerase chain termination sites. The percent modification represents the percent of bases in T7 DNA modified by AAF residues as standardized by the [^{14}C]AAF modified T7 DNA (see Materials and Methods). The right ordinate scale was established using 0% modification for the control DNA and 0.487% modification as measured for the [^{14}C]AAF DNA and their respective A_{305}/A_{260} ratios. (○) Experimental points from Table I; (▲) [^{14}C]AAF DNA: A_{305}/A_{260} plotted vs. chain termination sites per 1000 nucleotides in the DNA as calculated from the number average chain length reduction in double labeling experiment; (■) [^{14}C]AAF DNA: A_{305}/A_{260} vs. number of [^{14}C]AAF residues per 1000 bases calculated from the specific activity of the DNA.

sis. Thus, modification of DNA by AAF appears to cause premature termination of transcription by RNA polymerase.

From the reduction in number average chain length of RNA we have calculated the average number of chain terminating AAF hits per 1000 nucleotides in the DNA (Table I). These values are further plotted against the relative degree of AAF modification (A_{305}/A_{260}) in Figure 3. The graph shows a linear relationship between the extent of AAF modification (A_{305}/A_{260}) and the number of RNA chain terminating hits, thus indicating that there is no cooperative effect in the RNA chain terminating events produced by DNA-bound AAF.

In order to relate the A_{305}/A_{260} ratio to the actual percent modification by AAF, T7 DNA was modified with [^{14}C]-N-AcO-AAF. From the specific activity of the modified DNA (see Materials and Methods), we have calculated that AAF was bound to 0.49% of the bases. We have then plotted the A_{305}/A_{260} of this DNA vs. its actual number of [^{14}C]AAF residues per 1000 bases as well as vs. the chain terminating sites per 1000 bases, calculated, as for the unlabeled templates, from the observed chain length reduction of its transcription product. The measured percent modification of this DNA was used to establish the right-hand ordinate in Figure 3 and thereby provide an estimate of the percent modification of the unlabeled AAF-DNA samples. Such analysis indicates that almost all of the bound AAF residues cause premature RNA chain termination by the RNA polymerase.

We have previously shown that the predominant in vitro transcription product from T7 DNA is a 2.2×10^6 dalton RNA species (Millette et al., 1970). This fact facilitated analysis by polyacrylamide gel electrophoresis of the RNA produced from AAF modified T7 DNA (Figure 4). In agreement with the results from double labeling experiments, the gel profiles clearly show that the average molecular weight of the transcription product dramatically decreases with increasing AAF modification of the DNA. The 2.2×10^6 dalton RNA species disappears with the concomitant appearance of lower molecular weight RNA. This provides further confirmation of the chain terminating effect of AAF modification.

We have furthermore analyzed the kinetics of disappearance of the 2.2×10^6 dalton T7 transcription product on gels A, B, and C of Figure 4. A semilog plot of the relative amount of this RNA vs. the AAF dose (A_{305}/A_{260}) yields a linear relationship (Figure 5). This, together with the observation that chain termination is a linear function of drug dose (Figure 3), demonstrates that the RNA chain terminating function of bound AAF residues follows one hit kinetics.

To further elucidate the mechanism of RNA chain termination induced by AAF modification of T7 DNA, the following analysis (see Bräutigam and Sauerbier, 1974) was performed on the polyacrylamide gel electrophoresis profiles shown in Figure 4. First the molecular weight (M) of RNA in each gel slice was determined from the mobility of known markers. Then the relative number, N_i of RNA chains for each molecular weight class was calculated for each gel slice (see Materials and Methods). (In the subsequent discussion, number of RNA chains always means relative number.) We then see that in agreement with the initiation data of Figure 2B and Table I, the total number of RNA chains synthesized is conserved with increasing AAF modification: from a summation of gel fractions 1–45, the total relative number of RNA chains in gels A, B, and C corrected for the applied sample volume is found to be 56.3, 50.1, and 60.8, respectively. Then from the reduction in the number of 2.2×10^6 dalton RNA chains we calculate the average number of chain terminating AAF "hits," m, in the 6700 base pair transcription region from $m = -\ln (N_D/N_O)$, where N_D = number of 2.2×10^6 dalton chains (slices 4–7) in the gel of AAF-DNA transcripts and N_O = number of 2.2×10^6 dalton chains in control gel A. Then for the AAF-DNA B, $m = -\ln (5.00/14.08) = 1.04$. Assuming a random distribution of the AAF hits within the early transcription region, the distribution of the prematurely terminated RNA chains transcribed from AAF-DNA B may be calculated. All of the RNA chains that are lost from the 2.2×10^6 dalton size class should appear in the lower molecular

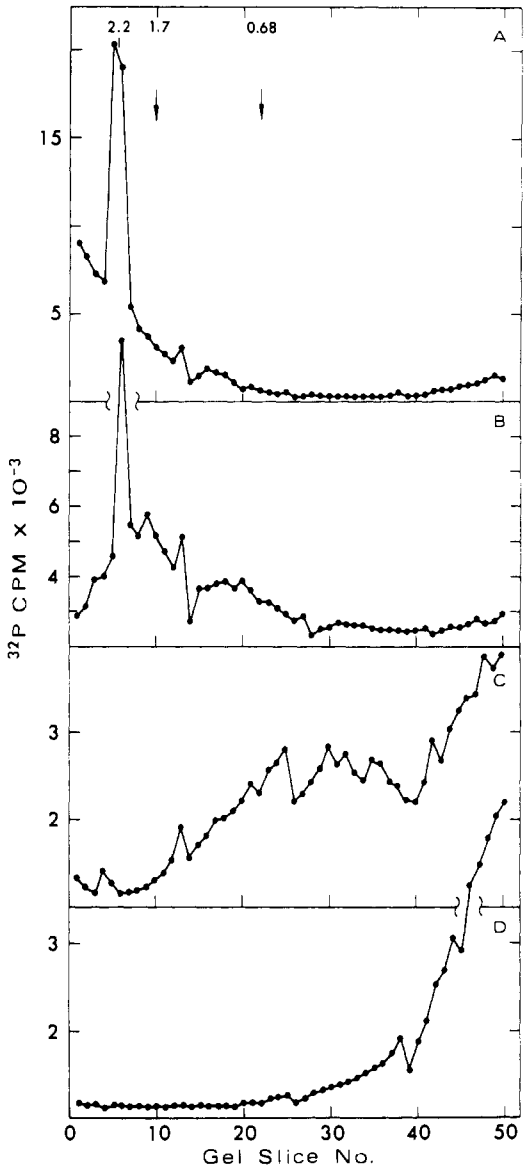

FIGURE 4: Polyacrylamide gel electrophoreograms of RNA synthesized from control and AAF modified T7 DNA. Reaction conditions are given under Materials and Methods with each 50-μl reaction mixture containing the four α-^{32}P-labeled ribonucleoside triphosphates at 35 cpm/pmol and 0.4 μmol/ml. Templates used were T7 DNAs A, B, C, and D (Table I) having increasing extent of modification by AAF. From reaction mixtures A, B, C, and D, 5-, 5-, 10-, and 15-μl aliquots, respectively, were used for gel analyses. Arrows and numbers indicate positions and molecular weights ($\times 10^{-6}$) of 18S, 28S L-cell RNA markers and the 2.2-megadalton T7 transcription product run in parallel gels.

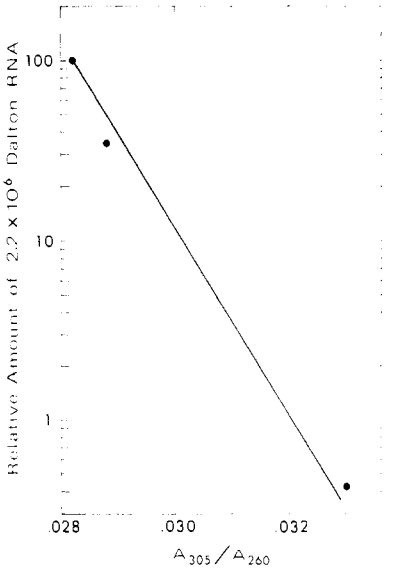

FIGURE 5: Relative amount of T7 primary transcription product synthesized as a function of increasing AAF modification of T7 DNA. From the gel profiles A, B, and C in Figure 4, the relative amounts of the 2.2-megadalton RNA synthesized were determined from a summation of radioactivity in gel slices 4–7 inclusive, normalizing these values to the same volume of reaction mixture in each case. Percent AAF modification is represented by the A_{305}/A_{260} ratio of the T7 DNA.

region of the gel. Then since the average number of hits, m, on DNA B = 1.04, we can calculate the fraction of the 2.2 $\times$ 10^6 dalton control RNA chains which will arise from DNA which received 0, 1, 2, and 3 AAF hits in the early gene transcription region. From Poisson distribution these are $p(0)$ = 0.355, $p(1)$ = 0.369, $p(2)$ = 0.192, and $p(3)$ = 0.067.

From the number of chains in the 2.2 $\times$ 10^6-dalton region of control gel A (14.06 chains), we can calculate the number of chains which we expect to appear in the 0.25 to 0.5 size RNA region (i.e., 0.55–1.1 $\times$ 10^6 daltons, fractions 14–26, inclusive) of gel B. The $p(1)$ class should have a rectangular distribution from zero to full chain length (2.2 $\times$ 10^6 daltons) and contain 0.369 of all the prematurely terminated RNA chains in gel B. The fraction of these expected in the 0.25–0.5 size region will be 13/42 (0.369) + 0.114 (where 13 = no. of fractions in the 0.55–1.1 $\times$ 10^6 dalton region and 42 = no. of fractions analyzed). The $p(2)$ and $p(3)$ of classes should fall in a broad distribution from 0.25 to 0.5 the size of the complete transcript and should amount to 0.192 + 0.067 = 0.259 of all the prematurely terminated chains. Thus, we expect to find an increase in the relative number of chains in the 0.25–0.5 size classes in gel B equal to (14.06)(0.114 + 0.259) = 5.24 chains. The actual number which appears is equal to

$$\sum_{14}^{26} B - (0.677)\sum_{14}^{26} A - (0.370)\sum_{1}^{3} A$$

where $\Sigma_{14}{}^{26}$ B equals the summation of number of RNA chains in fractions of 14–26 of gel B, etc. The value $(0.677)\Sigma_{14}{}^{26}$ A represents the contribution of the p(0) class of the 0.25–0.5 size RNA chains from gel A which will remain in gel B [assuming the DNA from which this size class arises will receive an average number of "hits" equal to $m \times \frac{1}{2}(\frac{1}{4} + \frac{1}{2})$ = (1.04)(0.375) = 0.39 "hits"; $e^{-0.39}$ = 0.677]. Since the RNA chains of greater than 2.2 $\times$ 10^6 daltons in the control should give rise to the same size distribution as do the 2.2 $\times$ 10^6 chains after AAF modification of the DNA, 0.37 represents the contribution of this class to the 0.25–0.5 size region. Thus the actual increase in chains in the 0.25–0.5 size region of gel B is 13.26 − 4.99 − 1.89 = 6.38 chains. This agrees fairly well with predicted values of 5.24 chains.

When a similar analysis is applied to gel C, we find that the average number of chain terminating "hits" is m = 4.9. The calculated influx of chains into the 0.25–0.5 size region of the gel is 3.44 and the actual increase in this region is found to be 4.01 chains, again both in good agreement.

From the foregoing analysis of the polyacrylamide gels we conclude that AAF modification of DNA causes RNA polymerase to terminate transcription with no reinitiation

and synthesis promoter distal to the AAF modification site. Initiation at or near AAF sites is unlikely since under our conditions of limiting DNA concentration (moles of enzyme/moles of DNA = 50) this would cause increased RNA chain initiation. Our data show this is not the case.

Discussion

The modification of DNA or RNA by AAF markedly alters their secondary structure as shown by increased nuclease susceptibility of double stranded RNA, decreased thermal stability and intrinsic viscosity of modified DNA, and lowered hybridization capacity of AAF-modified RNA (Levine et al., 1974). These studies support a base displacement model of AAF binding in which the modified bases are shifted out of the double helix while the covalently bound carcinogen is inserted. In this model there is restriction of rotation about the glycosidic bond (N_9–C_1) of guanosine due to the bulky substitution on C_8. The guanine is prevented from assuming its usual anti form in nucleic acid polymers resulting in a perturbation in the secondary structure around the modified base. In addition, the AAF residue is stacked in a coplanar configuration with the adjacent bases. This model predicts that either of several events might occur when RNA polymerase encounters a modified region in a double-stranded DNA template. The polymerase might (1) insert either the correct or incorrect base in the growing RNA chain, (2) skip the modified base or region and continue synthesis distal to the site of modification, or (3) completely stop causing a premature chain termination. Such events might be expected to have secondary effects on the rates of initiation and polymerization.

The results we have presented show that the major apparent effect of AAF modification of T7 DNA on transcription is an inhibition of the rate of RNA synthesis. At very high levels of modification (>0.72% of the bases modified), transcription is completely blocked. Analysis of the size of the RNA products by double labeling experiments and polyacrylamide gel electrophoresis shows that decreased rate of RNA synthesis on AAF modified templates is due to premature termination of transcription by RNA polymerase. By comparing the extent of modification by AAF with the number of chain terminating events we have shown that almost every base modified with a covalently bound AAF residue acts to cause chain termination.

The kinetics of transcription and initiation on the modified DNA show that when the polymerase reaches a site modified by AAF, it does not remain irreversibly bound to that site. If this were the case, both synthesis and initiation would stop at progressively earlier times with increasing extent of AAF modification. This was not observed.

AAF modification of DNA has very little effect on initiation of transcription. However, the initiation of RNA synthesis is slightly enhanced at the intermediate levels of bound carcinogen (ca. 0.2% of bases modified). This could possibly be due to a preferred modification of a GC-rich region at or adjacent to the polymerase binding site (Giacomoni et al., 1974; Dickson et al., 1974), and a resultant localized denaturation and facilitation of RNA polymerase binding and initiation. With more extensive modification there is a moderate decrease in initiation. This result is consistent with the relatively small target size of the initiation region as compared to the transcriptional unit being measured.

We have mentioned that in vivo administration of AAF or N-OH-AAF inhibits RNA synthesis in liver nuclei (Dawson, 1972; Grunberger et al., 1973). Furthermore, N-AcO-AAF is extremely toxic to mammalian cells in culture (L. Fink, unpublished observation). Both of these effects may be explained by the inhibition of transcription which we have observed with modified T7 DNA.

The mechanism of inhibition of DNA polymerase by AAF modification of DNA appears to be different. The in vivo results in bacterial systems indicate that AAF binding leads to a frame-shift type of mutation in repair deficient mutants (Ames et al., 1972). This suggests that DNA polymerase must be capable of either (i) reinitiating synthesis after the AAF modification with ligation of the reinitiated to terminated sequences, or (ii) reading past the modified base with deletion of the modified region. A detailed analysis of the capability of DNA synthesizing enzymes and repair enzymes to recognize and alter AAF-modified DNA in vitro will be of interest.

Acknowledgments

We express our thanks to Dr. Walter Sauerbier for his help in the mathematical analysis of the polyacrylamide gel data.

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The Assay and Isolation of DNA Rings Using an ATP-Dependent Endonuclease†

James R. Hutton and Charles A. Thomas, Jr.*

ABSTRACT: The ATP-dependent endonuclease from *Hemophilus influenzae* is relatively inactive on closed or open DNA rings, yet rapidly hydrolyzes single- or double-chained linear DNA. This enzyme in combination with an exonuclease (exo VII) has been shown to spare various circular DNA molecules including those having single-chain regions of significant length. However, rings containing single-chained regions are broken at a rate depending on the length of these regions. By admixing a linear DNA of alternate radiolabel, a simple assay for DNA rings has been developed. The application of this procedure to the assay of folded rings from *Drosophila* DNA is demonstrated.

While excellent methods are available for the isolation of covalently closed (superhelical) DNA rings (see Freifelder, 1971), the purification and assay of DNA rings that may contain interruptions (nicks) or single-chained regions (gaps) is more difficult especially if the rings are of variable molecular weight. The physical trapping of circular DNA molecules in agar gels is one possible method (Dean et al., 1973).

Here we present another method based on the observation that the ATP-dependent endonuclease from *Hemophilus influenzae* (Rd) does not attack single- or double-chained rings (Friedman and Smith, 1972a–c). Because this nuclease attacks linear single chains at one-tenth the rate observed with linear duplex DNA (Friedman and Smith, 1972c), we add another nuclease, exonuclease VII (Chase and Richardson, 1974), which removes nucleotides from both the 3′ and 5′ ends of single chains. The resulting mixture of nucleases spares duplex rings completely, yet rapidly reduces linear DNA to small oligomers. The spared material can be radioassayed by its Cl_3CCOOH insolubility or by its vastly different sedimentation rate. The fraction of DNA in rings as assayed by this nuclease method agrees with the same fraction determined by electron microscopy. While rings containing gaps are more resistant than linear DNA, they are broken at an observable rate which depends on the length of the single-chain regions. A similar approach has been developed by Mukai et al. (1973) for the isolation of plasmid DNAs employing an ATP-dependent nuclease from *Micrococcus luteus* which spares duplex rings but not single-chain DNA rings. To promote a more complete reaction, they also add an exonuclease, namely exo I.

Materials and Methods

DNAs. Labeled T_7 DNA was prepared by the method of Thomas and Abelson (1966); λ DNA by thermal induction of a stable lysogen (Malamy et al., 1972); SV40 DNA was a gift of George Fareed who prepared it by the method of Gelb et al. (1971). T_7 folded rings were prepared by first resecting the terminals with exonuclease III (Richardson et al., 1964) followed by annealing as described by Ritchie et al. (1967). *Drosophila* DNA was obtained from the stable *D. melanogaster* cell line K established by Echalier and Ohanessian (1970) grown in D22 medium supplemented with 10% heat inactivated fetal calf serum and 20 μCi/ml of [^{3}H]thymine or [^{32}P]phosphate. The confluent cell layers were washed twice with suspension buffer (0.03 *M* Tris–0.01 *M* EDTA (pH 8.3)). The washed cells were scraped from the falcon flask wall and suspended in a small volume of the suspension buffer. An equal volume of lysis buffer consisting of 0.03 *M* Tris, 0.01 *M* EDTA (pH 8.3), 2% sarcosyl, and 500 μg/ml of predigested Pronase was added to the cell suspension. The lysate was rocked gently and incubated at 36° for 12 hr, adjusted to $\rho = 1.45$ g/cm^3 with Cs_2SO_4 and banded by centrifugation at 40,000 rpm for 60 hr in a Beckman Type 65 rotor at 15°. The gradients were fractionated by dripping through a hole punched in the bottom of the tube. The fractions containing DNA were pooled and dialyzed against Tris-EDTA buffer (0.001 *M* EDTA–0.01 *M* Tris (pH 8.0)).

EM. Variations of the conventional Kleinschmidt method were employed (Lee et al., 1970).

Nucleases. Smith's ATP-dependent *Hemophilus* endonuclease (SAHN) was a gift from H. O. Smith and later prepared by the method described in Friedman and Smith

† From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received November 23, 1974.* This work was supported by National Science Foundation Grant GB-3118x and National Institutes of Health Grant GM-21136. J.R.H. is a Postdoctoral Fellow of the American Cancer Society.