Effect of carcinogenic adducts on transcription by T7 RNA polymerase

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ABSTRACT

The effect of modifying the T7 promoter-containing plasmid pDR100 with aminofluorene (AF), acetylaminofluorene (AAF), and benzo[a]pyrene (B[a]P) adducts on RNA synthesis by the T7 RNA polymerase was determined. We found that increasing levels of each of the three adducts caused a progressive decline in RNA synthesis, but that the inhibition produced by benzo[a]pyrene adducts was substantially greater than that caused by either AF or AAF adducts. Polyacrylamide gel electrophoresis analysis confirmed that the B[a]P adducts more strongly inhibited chain elongation since their presence resulted in the production of RNA fragments having average chain lengths very close to that predicted if each adduct permanently blocked the polymerase during transcription. We have also determined the effect of each type of adduct on the initiation of transcription and show that the T7 RNA polymerase initiates as efficiently on both AF or AAF-containing templates as it does on unmodified DNA, while B[a]P adducts were found to *increase* in the number of chain starts.

INTRODUCTION

The structure and reactivity of most chemical carcinogens is consistent with them mediating their effects by covalently interacting with DNA. It is well established that the vast majority of carcinogens are also mutagens (1) and most have been shown to be chemically reactive electrophiles or species which achieve reactivity through metabolic activation (2). Although the production of a point mutation at the site of the damage is the simplest explanation for how a chemical carcinogen induces its effect, there is a great deal of evidence which is not consistent with this mechanism (3, 4). This includes (i) the fact that *in vitro* transformation of rodent cells by chemical carcinogens can occur at a much higher frequency than exhibited by random point mutations, (ii) the higher resistance of human cells to *in vitro* transformation when compared to rodent cells while each is equally susceptible to mutagenesis, (iii) the lengthy latency period needed for the expression of the transformed state, (iv) the similarities between differentiation and carcinogenesis, and (v) the fact that tumors often display complex genomic changes in oncogenes and other cellular genes, including chromosomal translocations.

We are particularly interested in determining the effect on transcription of DNA modified by carcinogenic adducts using a well-characterized *in vitro* system. This system is comprised

of double-stranded DNA containing either N-(guanine-8-yl)-2-aminofluorene (AF-dGuo adduct), N-(guanine-8-yl)-2-acetylaminofluorene (AAF-dGuo adduct), or 2'-deoxy-N²-(7,8,9,10)-tetrahydro-7 β ,8 α ,9 α -trihydroxybenzo[a]pyrene-10-yl guanosine ((+) *anti*-BPDE-dGuo, BPDE I-dGuo) adducts, cloned T7 promoters and the highly purified RNA polymerase isolated from bacteriophage T7.

N-2-Acetylaminofluorene (AAF¹) is a potent rat liver carcinogen which, after metabolic activation, can bind to DNA through O-acetyl or sulfate derivatives (5). Activation by sulfate conjugation results in the binding of the AAF moiety at the C8 or N2 positions in guanine; O-acetylation introduces the 2-aminofluorene moiety to C8 of guanine through the arylamine nitrogen, which is the major adduct formed in all tissues studied so far (5). Treatment of double stranded DNA *in vitro* with N-acetoxy-2-acetylaminofluorene results predominantly in the acetylated adduct at C8 (6, 7), whereas N-hydroxy-2-aminofluorene gives the deacetylated adducts (8, 9). The effect that each of these adducts has on the conformation of DNA molecules has been extensively studied. For the AAF adduct, two related models (termed insertion-denaturation (10) and base displacement (11)) have been proposed which suggest that there is a substantial conformational change induced near the site of adduct binding. Both models predict that the DNA is locally denatured at the adduct site, with the guanine rotated in the *syn* conformation and the AAF moiety inserted into the DNA helix. However, in the case of the deacetylated AF adduct, there appears to be less severe distortion induced in the duplex DNA helix (termed the outside binding model (12)) such that the AF residue resides in the major groove of a normal B-helix. These conformational differences seems to correlate well with the differences in their biological activities. Thus AAF adducts are more rapidly excised from rat liver DNA than AF adducts (13); AAF adducts on ϕ X174 DNA inhibit transfections to a much greater extent than AF adducts (14); and AAF adducts generate mostly frameshift mutations (15) while AF adducts produce mainly point mutations (16).

Benzo[a]pyrene (B[a]P), a ubiquitous environmental pollutant, becomes a potent mutagen and carcinogen after metabolic activation by the cytochrome P-450 mixed function oxidase system. Recent studies indicate that ($\pm$)-7 β , 8 α -dihydroxy-9 α , 10 α -epoxy 7, 8, 9, 10-tetrahydroxybenzo[a]pyrene (BPDE I) is the ultimate carcinogenic and mutagenic form of B[a]P (17, 18). The major adduct formed *in vitro* (19, 20) and *in vivo* (20) results from the binding of the 2-amino group of guanosine to the C-10 position of B[a]P diol epoxide I. This adduct represents 80-90% of all stable adducts formed (19, 20) and is implicated in the mutagenic (21) and carcinogenic (22) effects of B[a]P. There are results indicating that the binding of diol epoxide to DNA distorts the helix but the exact conformation is still disputed (23-27).

The effect of these adducts on DNA synthesis has been the subject of numerous investigations (27, 28). For the most part, these bulky type adducts have been shown to

block the polymerase at the site of the adduct, with bypass occurring slowly or not at all. The effect on RNA synthesis has been less well studied but seems to be comparable with the DNA synthesis results. Both chain elongation and initiation by *E. coli* RNA polymerase was inhibited *in vitro* by B[a]P adducts (29), while AAF adducts were shown to inhibit RNA synthesis in rat liver nuclei (30), on purified rat liver DNA (31), or *in vitro* by *E. coli* RNA polymerase (32).

In this paper we have used a T7 promoter-containing plasmid (pDR100) modified to varying extents with AF, AAF, or B[a]P adducts to determine the precise effect of these adducts on RNA elongation and initiation by the T7 RNA polymerase. We find that all three adducts inhibit synthesis to some extent, but that the B[a]P adduct is by far the most inhibitory. Further, we show that there is little difference between the effect of AF or AAF adducts on RNA synthesis and that neither significantly alters the number of RNA chain initiations compared with that determined on unmodified DNA. B[a]P adducts, on the other hand, cause a substantial *increase* in the measured level of initiation of RNA chains.

MATERIALS AND METHODS

DNA and Nucleotides

Plasmid pDR100 (33) (T7 67.5-74.1) was isolated after chloramphenicol amplification as described (34) using a CsCl gradient to separate the nicked and supercoiled forms of the DNA. Unlabeled nucleotides were purchased from P. L. Biochemicals. Radioactive nucleotides were obtained from ICN Radiochemicals.

T7 RNA Polymerase

T7 RNA polymerase (specific activity 124,000 units/mg) was purified from the cloned T7 gene 1 in plasmid pBR322 as described (35).

Other Materials

Sephadex G-50 was obtained from Pharmacia. [*ring*-³H]-N-hydroxy-2-aminofluorene (60 mCi/mmol) (33), [*ring*-³H]-N-acetoxy-N-acetyl-2-aminofluorene (36 mCi/mmol) (36), and [*ring*-³H]-N-acetoxy-N-trifluoroacetyl-2-aminofluorene (60 mCi/mmol) (37) were synthesized and characterized as described. [*ring*-³H] (±)-7β, 8α-dihydroxy-9α, 10α-epoxy 7, 8, 9, 10-tetrahydrobenzo[a]pyrene (1.38 Ci/mmol) was purchased from Midwest Research Institute. NCS (tissue solubilizer) was purchased from Amersham. All reagents and nucleotide solutions were prepared using diethylpyrocarbonate-treated water and were subsequently sterilized.

Modification reactions with plasmid DNA

Plasmid DNA containing AF adducts were prepared by the reaction with either [*ring*-³H]-N-hydroxy-2-aminofluorene (60 mCi/mmol) (28) or [*ring*-³H]-N-acetoxy-N-

trifluoroacetyl-2-aminofluorene (60 mCi/mmol) (38). No differences were found using the DNAs prepared by either of these methods.

pDR100 (100 µg/ml) was reacted with freshly prepared [*ring*-³H]-N-hydroxy-2-aminofluorene (60 Ci/mmol) in 10 mM citrate (pH 5.5), 1 mM EDTA, in the presence of 20% ethanol. Reactions were carried out in the dark under nitrogen atmosphere for one hour. Each reaction mixture was then extracted once with phenol, four times with ether, and the DNA was then twice precipitated with ethanol. The number of adducts formed per molecule was calculated from the ratio of radioactivity to absorbance at 260 nm.

Modification of DNA with [*ring*-³H]-N-acetoxy-N-trifluoroacetyl-2-aminofluorene (60 mCi/mmol) results only in the formation of the deacetylated aminofluorene adducts at the C8 position of guanine. In a typical reaction pDR100 (100 µg/ml) was incubated with 10-12 µg/ml of freshly prepared [*ring*-³H] N-acetoxy-N-trifluoroacetyl-2-aminofluorene (60 µCi/µmol) under an argon atmosphere in a solution containing 8 mM citrate (pH 7.0) and 20% ethanol. Reactions were carried out in the dark for times ranging between 15 and 90 minutes, depending on the level of modification required. Following modification, the reaction mixture was extracted 10 times with diethyl ether. No radioactivity was detectable in the organic phase following the tenth extraction. The DNA in the resulting aqueous phase was precipitated three times with ethanol. The number of adducts formed per molecule of pDR100 was determined as described above.

The reactions of plasmid pDR100 with [*ring*-³H]-N-acetoxy-N-acetyl-2-aminofluorene (36 mCi/mmol) and [*ring*-³H] (±)-7β, 8α-dihydroxy-9α, 10α-epoxy 7, 8, 9, 10-tetrahydroxy-benzo[a]pyrene (1380 mCi/mmol), and subsequent purification was done as described previously (39). The extent of modification was determined as described above.

RNA Synthesis Assays

RNA synthesis by T7 RNA polymerase was measured in a reaction (final volume 0.05 ml) containing 40 mM Tris·HCl pH 8.0, 10 mM MgCl₂, 10 mM dithiothreitol, 50 mM NaCl, 12.5 µg of bovine serum albumin, 0.4 mM ATP, GTP, CTP and [³H] UTP (30-50 cpm/pmol), and 1 nm of modified and unmodified DNAs. Reaction mixtures were incubated with increasing amounts of enzyme in the dark at 37°C for 10 minutes and were stopped by the addition of 3 ml of ice-cold 100 mM Na₄P₂O₇ in 1N HCl. Time course reactions were incubated with 5 units of enzyme and stopped at intervals of 10 minutes as described above. The acid insoluble products were filtered through Whatman GF/C filters, washed three times with acid solution and once with 3 ml of cold 95% ethanol. Acid insoluble radioactivity was then determined in a Beckman LS 7500 liquid scintillation counter.

For double labeling experiments, [³H] UTP (30-50 cpm/pmol) and [³²P] GTP (2000-2500 cpm/pmol) were used as labeled substrates. RNA synthesis was carried out as

described above with 10 units of RNA polymerase. The reactions were stopped by the addition of 5 μ l of 0.5 M EDTA and the aqueous phase recovered after one extraction with an equal volume of phenol. The unincorporated labels were removed by passing the reaction mixtures through a G-50 column as described (40). The fractions containing the labeled products were then collected, precipitated with acid, filtered and counted as described above.

Polyacrylamide Gel Electrophoresis

RNA synthesis was carried out as described above except that the radionucleotide used was [α - 32 P] GTP (800-1000 cpm/pmol) and synthesis was done with 15 units of T7 RNA polymerase. Reactions were stopped by the addition of 5 μ l of 0.5 M EDTA, and the aqueous phase extracted with equal volume of phenol. The unincorporated label was removed by passing through a Sephadex G-50 spun column as described (34). An aliquot of the reaction was loaded onto a denaturing (7 M urea) 5% polyacrylamide gel and electrophoresed until the bromophenol blue dye migrated to the end of the gel. Autoradiography was carried out at -70 $^{\circ}$ C using Kodak XAR-5 film and a Dupont Cronex Lightning Plus intensifying screen.

Processing of agarose gel for scintillation counting

Modified DNA (0.2 μ g) was electrophoresed in 1% agarose gel in TBE (Tris-borate-EDTA) buffer as described (34). The gel was stained with ethidium bromide (1 μ g/ml) and 1 mm slices of the lane were then cut and transferred to scintillation vials. NCS (Amersham) tissue solubilizer (5 ml) was then added and the mixtures incubated overnight at 50 $^{\circ}$ C. The vials were cooled and, after adding 5 ml of fluor, radioactivity was determined in Beckman LS-7500 liquid scintillation counter.

RESULTS

Preparation and characterization of modified DNA templates

Duplex plasmid DNA was modified with tritiated aminofluorene, acetylaminofluorene, or benzo[a]pyrene as described in the Material and Methods section. Following purification, each DNA sample was analyzed by trifluoroacetic acid hydrolysis and an HPLC analysis comparison of [3 H]-containing adducted bases with authentic standards as described (38) (not shown). In addition each sample was analyzed by agarose gel electrophoresis in order to ensure the integrity of the DNA sample and to confirm the covalent association of the carcinogen with the DNA. Following electrophoresis and ethidium bromide staining, the gel lanes were separated, sliced and the tritium in individual slices determined. As shown in Fig. 1, following modification with B[a]P to a level of 5 adducts per DNA molecule, the majority of the DNA (>90%) remained supercoiled, and all of the detectable radioactivity in the gel slices was associated with the ethidium bromide-stained DNA. Even with modification levels as high as 50 adducts per molecule, we did not observe more than 10-15% of the DNA

Fig. 1. Analysis of [^3H]-labeled B[a]P-modified pDR100 DNA. DNA containing 5 [^3H]-labeled B[a]P adducts per molecule was electrophoresed on a 1% agarose gel, stained with ethidium bromide, sliced, and the radioactivity present in each slice determined as described in "Materials and Methods." A photograph of the stained gel is shown above the graph of the counted slices.

becoming nicked. Similar analyses were carried out with the AF and AAF-modified DNA samples. In the case of the AAF modification, we found that when the adduct level approached 50 adducts per molecule, as much as 50% of the DNA became nicked (data not shown). With the AF-modified DNA, however, even at maximum levels of modification (80 adducts/molecule) less than 10% of the DNA became nicked.

RNA synthesis on modified templates

The plasmid pDR100 is a pBR322 derivative which contains a 2625 BP insert from T7 DNA. This T7 DNA contains the sequences from position 67.5 to 74.1 which includes the class III T7 $\phi 13$ promoter. The T7 RNA polymerase is able to efficiently initiate transcription from this promoter giving RNA chain lengths which can equal or exceed the size of the DNA template (41). Transcription on the nicked form of this DNA is reduced by approximately 20-30% compared with the supercoiled form due to the increased affinity of the RNA polymerase for supercoiled DNA (41) and to the blockage of the polymerase at the site of the nick.

We have compared synthesis on this DNA template modified with either AF, AAF or B[a]P adducts as a function of the number of adducts per molecule (Fig. 2). It is evident from this data that, of the three types of lesions, the B[a]P structure had a significantly greater effect

Fig. 2. RNA synthesis on AF, AAF, and B[a]P-modified templates. Plasmid pDR100 containing 1, 10, 17 AF adducts/molecule, 3, 13, 20 AAF adducts/molecule and 5, 10, 23 B[a]P adducts/molecule was used as templates for RNA synthesis by the T7 RNA polymerase (5 units). The levels of RNA synthesis in each case were determined in triplicate as described in "Materials and Methods."

on synthesis than either the AF or AAF adducts. For example, when the template contained either 3 AF or 3 AAF adducts per molecule, synthesis was reduced by about 50%, while 5 B[a]P adducts resulted in a 90% reduction in RNA synthesis. It is also interesting to note that the AF and AAF adducts had similar inhibitory effects on RNA synthesis at each of the levels of modification. This is somewhat surprising since these adducts have been suggested to have vastly different structures in DNA (10, 11).

The results of a time course of RNA synthesis using comparably modified DNA containing either AF, AAF or B[a]P adducts is shown in Fig. 3. For each template, RNA synthesis was linear for the course of the 40 min reaction. The rate of synthesis on DNA containing 11 AF or 13 AAF adducts per molecule was 51% (45 pmol of nucleotide/min) of that found using a native (unmodified) template, while synthesis on DNA templates containing 10 B[a]P adducts per molecule was three-fold lower (14% of native, 12 pmol of nucleotide/min). This is consistent with the data presented in Fig. 2 which shows that the RNA polymerase was much more sensitive to the presence of B[a]P adducts in DNA than either AF or AAF adducts. The fact that these reactions remained linear over the full 40 min reaction time suggests that the polymerase is not being permanently inhibited or sequestered by the modified template.

Fig. 3. Time course of RNA synthesis on AF, AAF, and B[a]P-modified DNA. Plasmid pDR100 containing 11 AF, 13 AAF, and 10 B[a]P adducts per molecule were used as templates for RNA synthesis by the T7 RNA polymerase (5 units). The levels of RNA synthesis in each case were determined in triplicate as described in "Materials and Methods."

Although the polymerase was in excess in these experiments (if one assumes that 100% of the polymerase molecules in the preparation are active) we found similar results under conditions where the template is in excess (data not shown).

Two possible interpretations of these data are that either (i) the adducts inhibit synthesis by blocking the polymerase, causing it to fall off the template and reinitiate synthesis on a new promoter-containing molecule or (ii) the adducts simply slow the progress of the polymerase but do not act as absolute blocks to elongation. We have attempted to distinguish these two possibilities by determining if the adducts cause an increase in the number of RNA chains initiated, even though the total amount of RNA synthesized is reduced, by measuring the RNA chain lengths for synthesis on modified templates. If the adducts were simply slowing the rate of synthesis, then the number of initiation events and the chain length of the product RNA should be similar on native and modified templates. On the other hand, if the adducts block transcription, then the RNA synthesized should have a shorter chain length, while the number of initiations might be expected to increase.

Initiation of transcription on modified templates

In order to measure the number of initiation events during RNA synthesis by the T7 RNA polymerase, we have quantitated the incorporation of [γ - 32 P] GTP into RNA chains from the ϕ 13 promoter. This is a sensitive assay for the initiation of RNA synthesis because the first

TABLE I Effect of AF, AAF, and B[a]P adducts on transcription by T7 RNA polymerase.

Template	Synthesis ^a (nmol)	Initiation ^a (pmol)	RNA chain length ^b	Adduct-induced stops ^c
pDR100	1.8	0.32	5600	--
pDR100 (nicked)	1.5	0.35	4300	--
AF-modified ^d	0.60	0.32	1900	1.9
AAF-modified ^e	0.46	0.36	1300	3.3
B[a]P-modified ^f	0.24	0.48	500	10

^aAverage of two experiments. In all cases the standard deviation was less than 10%.

^bCalculated from the ratio of initiation to synthesis.

^cDetermined as described (32) (see text).

^dThis template contained 17 AF adducts per double-stranded DNA molecule.

^eThis template contained 20 AAF adducts per double-stranded DNA molecule.

^fThis template contained 23 B[a]P adducts per double-stranded DNA molecule.

nucleotide incorporated into this transcript is GTP; GTP incorporated internally will have lost the ³²P label. Table I shows the amount of RNA synthesis and chain initiations measured simultaneously for transcription on supercoiled or nicked pDR100, and AF, AAF, or B[a]P-modified DNA. The ratio of initiation events to total RNA synthesis gives the average chain length for synthesis on each of these templates. We have included both the supercoiled and nicked forms of the unmodified DNA as reference points to confirm the anticipated inhibitory effect of nicking on synthesis. If every molecule contained a single phosphodiester bond interruption—which should block the polymerase at the site of the nick—then synthesis should be reduced by 25% since the nick should be randomly located and could be in either the template or displaced strand. This is confirmed in Table I from an analysis of both the extent of RNA synthesis and the average chain lengths for the fully nicked material, each of which was reduced by approximately 25%. Because each of the modified templates used in this experiment were no more than 10% nicked, this factor should not have a significant contribution to the reduction of synthesis observed.

Using templates containing either 17 AF, 20 AAF, or 23 B[a]P adducts per molecule resulted in the expected reduction in the total extent of RNA synthesis (Table I). Although RNA elongation was reduced on all three templates, the number of initiation events on AF-modified DNA was approximately the same as that found with native DNA. However, even though there was a reduction in total RNA synthesis on the AAF and B[a]P-modified DNA, we observed an increase in the levels of initiation on both of these templates (Table I). For the AAF-modified DNA this increase was 13% while the B[a]P modification caused a

Fig. 4. Polyacrylamide gel of synthesis by T7 RNA polymerase on native and modified pDR100. RNA synthesis by the T7 RNA polymerase (15 units) on unmodified nicked circular pDR100 DNA and AF, AAF and B[a]P-modified DNA was carried out as described in "Materials and Methods" using an [α - 32 P] GTP label. The 32 P-labeled products were electrophoresed on a 5% denaturing polyacrylamide gel and subsequently autoradiographed as described in "Materials and Methods." The numbers above each lane represent the number of adducts per molecule present on each template.

more substantial increase of 50%. It must be noted that we have no way of determining what contribution the presence of an adduct in the promoter sequence has on the number of RNA chains initiated. At a level of 20 adducts per molecule, approximately 5% of the promoters should contain an adduct. If these promoter-bound adducts inhibit the binding of the polymerase, then these templates would not be used for RNA synthesis and would not contribute to the measured levels of initiation or elongation. However, it is unlikely that a

reduction in the total number of available template molecules by 5% would alter the results presented here unless the polymerase were being irreversibly bound to the adduct-containing promoter, a circumstance which seems improbable.

It is possible to directly determine the average chain length of the RNA synthesized by calculating the ratio of initiation to elongation for each of these templates (Table I). It is apparent that the average chain length for the RNA synthesized on the modified templates was reduced compared with either native supercoiled or nicked DNA and that there was a hierarchy observed for this effect—the RNA product was shortest on the B[a]P-modified template and longest on the AF-treated DNA. These data suggest that the B[a]P adduct was much more efficient at blocking the polymerase than either the AF or AAF adduct and that, of the two aromatic amine adducts, the AAF structure had the greater effect. From the data shown in Table I and using the formula (32):

$$\text{stops} = (\text{chain length native} / \text{chain length modified}) - 1$$

we calculate that there are 2 adduct-induced stops for the AF-modified template, 4 to 5 stops for the AAF-modified template, and 10 for the B[a]P-modified DNA.

Polyacrylamide gel analysis of RNA synthesis

We have utilized polyacrylamide gel electrophoresis of the [α - ^{32}P] UTP labeled RNA product to visualize the effect of these adducts on RNA synthesis. Shown in Fig. 4 is an autoradiogram which compares synthesis on templates modified to various levels with AF, AAF, and B[a]P. As was found for the incorporation of nucleotides into acid insoluble RNA, RNA synthesis, measured by the intensity of the radiolabeled bands in the gel, was reduced on modified templates with increasing level of modification. The effect was greatest for the B[a]P-modified template and in this case many short RNA transcripts were observed, the intensity of which increased with increasing levels of modification of DNA. These discrete bands were not observed with either the AF or AAF-modified DNA containing similar levels of adducts. We estimate from densitometer scans of these gels that the average chain length for synthesis on templates containing either 17 AF, 20 AAF or 23 B[a]P adducts is 2000, 1250, and 475, respectively. These numbers show good agreement with those calculated from the ratio of chain initiations to chain elongation.

DISCUSSION

We have used the well characterized T7 *in vitro* system to study the effect of DNA modification by bulky carcinogenic adducts on RNA transcription. This system takes advantage of the fact that the T7 RNA polymerase is a relatively simple enzyme comprised of a single polypeptide chain. It can be isolated in large amounts from *E. coli* cells containing

plasmids bearing the cloned T7 RNA polymerase gene, as can cloned T7 RNA polymerase promoters. This system, comprised of purified T7 RNA polymerase and cloned T7 single promoter-containing templates, is, therefore, a convenient source of defined transcripts which can be used to determine the precise effects of various forms of DNA damage on transcription.

For the initial studies presented here, we have used templates randomly modified with three well-characterized carcinogenic adducts—aminofluorene (AF), acetylaminofluorene (AAF) and benzo[a]pyrene (B[a]P). These adducts were chosen because they are among the best characterized in terms of adduct structure and their effect on DNA metabolism. In addition, these particular adducts comprise an interesting set for comparison based on their reported structures and properties. Both the AF and AAF adducts have been shown to react at the C8 position of guanine, differing in chemical structure only by the presence (AAF adduct) or absence (AF adduct) of a nitrogen-linked acetyl group. However, these two adducts seem to result in strikingly different structures in the DNA molecule. While the AF structure has been shown to be relatively nondistorting to the DNA helix, causing little or no local denaturation (12), the AAF structure apparently causes a significant degree of distortion, resulting in several nucleotides near the adduct becoming unpaired (10, 11). In fact, there is evidence that in the AAF structure the modified guanine has rotated around the glycosidic bond causing the base to adopt a *syn* conformation. Finally, these aryl amine adducts form an interesting contrast with the B[a]P adduct since it is, instead, linked to the base pairing portion of the helix, predominantly bound to the N2 position of guanine (19, 20).

We have found that the inhibition of RNA synthesis on these modified templates increased as the level of modification increased for each type of damage studied. However, the effect of the B[a]P adduct was substantially greater than that observed for either the AF or AAF, while the inhibition by the two aryl amine adducts was approximately the same (Fig. 2, 3). We found that the rate of synthesis on each of these templates was linear throughout the course of the reaction, even under conditions when the polymerase was limiting. The mechanism causing this inhibition of synthesis was most likely the result of either the polymerase being (i) completely blocked by the adduct or (ii) simply slowed at the adduct site. If the former were true, then one might predict that although total RNA synthesis is reduced on the modified template, initiation events would increase. This is exactly the result observed for synthesis on B[a]P-modified templates. In this case, we found that while total RNA synthesis, as measured by the incorporation of [³H] UTP into acid-insoluble RNA, dropped substantially, the incorporation of [γ -³²P] GTP, which specifically measures initiation events, increased by over 50%. This same effect was not observed in the case of the AF adducts. Here, although synthesis was reduced, the rate of initiation of new RNA chains was very similar to that observed on unmodified templates, suggesting that AF adducts may have

slowed elongation but may not have provided absolute blocks to the polymerase. In the case of the AAF modified template, we observed results intermediate between the AF and B[a]P examples. On this template, initiation increased by just 12%, suggesting that this adduct blocked the polymerase to a greater extent than the AF adduct, but not as effectively as the B[a]P structure.

We have also quantitated the number of adduct-induced chain terminations for each template from the average chain lengths determined either from the ratio of initiation to elongation synthesis or by polyacrylamide gel analysis. For the templates containing either 17 AF or 20 AAF adducts per molecule it is clear from the polyacrylamide gel that there are few observable short chain bands and that the majority of the RNA synthesized is near full-length transcripts. We calculate from the ratio of initiations to elongation that the average chain length for the AF-modified template was slightly less than 1900 nucleotides, while in the AAF case the average chain length was just under 1300. This corresponds to 2 and 4.5 adduct-induced chain terminations for the AF and AAF-modified templates, respectively. From this data, it appears that trans-lesion synthesis occurred efficiently with either template, although the AAF adduct appears to block synthesis with approximately twice the frequency as the AF adduct. In light of the reported greater distortion to the DNA structure caused by the AAF adducts, this result seems reasonable. We have substantial evidence using either randomly modified (28) or site-specifically modified DNA² that a variety of DNA polymerases efficiently bypass the AF structure during synthesis on a single-stranded template.

The ease with which the T7 RNA polymerase can bypass AAF adducts is somewhat surprising and particularly interesting based on prior *in vitro* DNA synthesis studies or on *in vivo* experiments carried out by various groups. Using single-stranded templates modified with AAF adducts, Moore *et al.* have shown that both DNA polymerase I and AMV polymerase rarely if ever synthesize past this lesion. In addition, several studies have predicted that a single unrepaired AAF adduct can be lethal in both a single or double stranded genome (14, 42, 43). Therefore, it is important to address the question whether the substantial bypass RNA synthesis observed with this lesion reflects the *in vivo* situation or simply reflects an artifact of the *in vitro* reaction, such as slippage at the adduct site or strand switching on the displaced strand.

There are several indications that the effects we observe do, in fact, reflect trans-lesion synthesis past an AAF adduct. First, the data obtained for the AF or AAF adducts contrast sharply with that obtained for RNA synthesis on the B[a]P-modified DNA templates. In this case, adduct-induced chain terminations are clearly evident in the polyacrylamide gel obtained from RNA synthesized on a template containing 23 adducts per molecule (Fig. 4). We estimate from a densitometer scan of this gel that the average chain length is approximately

475 nucleotides. These data agrees quite well with that obtained from the ratio of initiations to elongation which indicate that the average chain length is approximately 500 nucleotides. We calculate that this length RNA transcript for this template corresponds to 10 adduct-induced chain terminations suggesting that the polymerase is bypassing an adduct approximately half the time. However, since these templates contain adducts in both the template and displaced strands, it is possible that these adducts are absolute blocks to the RNA polymerase, just as they completely block DNA polymerase (44), when they are situated in the template strand but that trans-lesion synthesis occurs if the adduct is on the displaced strand. (We are currently testing by constructing templates specifically modified in either the template or displaced strands.) Second, the polyacrylamide gel of synthesis on AF modified templates shows that the vast majority of synthesis extends to the end of the template. Had some form of artifactual slippage occurred or had the polymerase been encouraged to switch strands at the adduct site, a substantial amount of shorter transcripts would have been observed in Fig. 4. Finally, it is not entirely unreasonable that different effects could be observed for synthesis by DNA *versus* RNA polymerases on templates containing the same adduct. Because all known prokaryotic polymerases contain a 3'→5' exonuclease proofreading activity, while RNA synthesis is not known require this editing feature, it may be reasonable that lesions, such as the AAF adduct, which are not bound to the Watson-Crick basepairing region, do not inhibit RNA synthesis to the same extent as DNA synthesis is blocked.

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¹Abbreviations: AF, 2-aminofluorene; AAF, N-2-acetylaminofluorene; B[a]P, benzo(a)pyrene.

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