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NUCLEASE P_1-MEDIATED FLUORESCENCE POSTLABELING ASSAY OF AAF MODIFIED DNA MODEL d(TACGTA) AND CALF-THYMUS DNA

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SUMMARY: Fluorescence postlabeling assay for DNA damage combines enzymatic digestion of modified DNA to nucleoside monophosphates and fluorescence postlabeling. However, to facilitate the quantitative release of bulky adducts that are not readily obtained as mononucleotides, a different mechanism is essential. In order to test the application potential of nuclease P_1-mediated fluorescence postlabeling to assay bulky adduct, d(TACGTA) was reacted with N-acetoxy-2-acetylamino-fluorene. The major product, characterized by nmr as AAF adduct of the guanine moiety at the C-8 position, was used as a DNA model. Nuclease P_1 digestion of the modified oligomer excised the adduct in two forms (3:1) which were identified as AAF modified d(pGpT) and dpG respectively by co-chromotography with authentic markers. Fluorescence postlabeling assay of AAF modified d(TACGTA) detected both forms of the excised adduct. The application of the overall procedure to assay AAF modified calf-thymus DNA demonstrated that the extension of fluorescence postlabeling technique from the mononucleotide to the dinucleotide version expands the scope of the assay. © 1991 Academic Press, Inc.

We developed a novel assay for DNA damage by combining DNA digestion with fluorescence postlabeling (1). Briefly, the modified DNA is digested with DNase I and snake venom phosphodiesterase (SVPDE) yielding 5′-monophosphate of normal and modified bases. The modified nucleotides are enriched by HPLC and labeled with dansyl chloride through the 5′-phosphoramidate linkage with ethylenediamine. The dansylated nucleotides are then analyzed by HPLC using fluorescence detection. Two specifically modified nucleotides namely, cis-Tmp glycol and 8-OH dGmp have been detected in X-irradiated calf-thymus DNA by fluorescence postlabeling assay (2,3). In the case of 8-OH dGMP, the content of the modified nucleotide was quantitated and found to increase linearly in a dose dependent manner.

The treatment of modified DNA with micrococcal nuclease (MN) and bovine spleen phosphodiesterase (BSPDE) generates 3′-monophosphates of normal and modified bases (4). We observed that fluorescence postlabeling assay, initially

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developed for 5′-digest, is also applicable to 3′-DNA digest (5). However, for more quantitative release of those DNA adducts that are not readily obtained as mononucleotides (6), a different mechanism of DNA digestion is essential. Randerath et al. (7) reported that when DNA containing bulky adducts are digested with nuclease P_1 at pH 5, normal nucleotides are released as 5′-monophosphates, pN whereas adducts are excised as 5′-phosphorylated dinucleotide pXpN (X = modified base).

Nuclease P_1 digestion of modified DNA has potential to offer two distinct advantages to assay DNA damage by fluorescence postlabeling. First, HPLC resolution of a modified nucleotide from the normal mononucleotides, pN is better when the modified nucleotide is excised in a dinucleotide, pXpN rather than mononucleotide form, pX. As a result, prior to labeling, enrichment of the modified nucleotide becomes more efficient. Secondly, the excised modified dinucleotide is 5′-phosphorylated and therefore, after enrichment from the normal nucleotides, can be labeled directly with dansyl chloride without further enzymatic manipulation. The dinucleotide version of ^{32}P-postlabeling assay requires 5′-dephosphorylation of the modified dinucleotide with acid phosphatase to introduce the radio label at the 5′ end with γ-^{32}P labeled ATP and polynucleotide kinase (7). In order to test the application of nuclease P_1-mediated fluorescence postlabeling to assay bulky adduct, a deoxyhexanucleotide d(TACGTA) was synthesized as a DNA model and reacted with N-acetoxy-2-acetylaminofluorene (N-acetoxy-AAF). Initially, the modified hexamer was used as a substrate for nuclease P_1. The procedure was then extended to AAF modified calf-thymus DNA. This report describes the nuclease P_1-mediated fluorescence postlabeling assay of AAF modified d(TACGTA) and calf-thymus DNA.

MATERIALS AND METHODS

Chemicals. Protected mononucleotide phosphotriesters and protected nucleosides for oligomer synthesis were obtained from Gallard-Schesinger Biochemicals. N-Acetoxy-AAF was obtained from National Cancer Institute's Chemical Repository. The enzyme nuclease P_1 from Penecillium Citriums and phosphodiesterase I from Crotalus Adamanteus Venom were purchased from Sigma Chemical Co. and Pharmacia respectively. Dansyl chloride, ethylenediamine and 1-methylimidazole were products of Sigma. 1-(3,3-Dimithylaminopropyl)-3-ethylcarbodiimide hydrochloride was obtained from Aldrich Chemical Co.

The nmr spectra were acquired by a Bruker WP200 spectrometer, 200 MHz for proton and 50 MHz for carbon-13.

Methods. The synthesis of d(TACGTA), d(pGpT), their AAF modified derivatives and the fluorescence postlabeling of the modified d(pGpT) with dansyl chloride were carried out following the procedures reported earlier (8,1). Calf thymus DNA was reacted with N-acetoxy-AAF and the modified DNA was digested with nuclease P_1 following reported procedures (9,10). The HPLC conditions for the analysis of various nucleotides are included in the figure legends.

RESULTS AND DISCUSSION

The hexadeoxynucleotide d(TACGTA) was synthesized in solution-phase by a modified phosphotriester technique and reacted with N-acetoxy-AAF as reported earlier (8). The hexanucleotide d(TACGTA) was characterized by ^{1}H nmr both before and after modification (8). The proton nmr of the AAF modified hexamer was fairly complex. The resolution of the downfield base protons was difficult due to the overlapping of some of the fluorene resonances. The loss of the proton resonance at 7.9 ppm due to the G H-8 in the hexamer after modification shows, however, that the covalent attachment of AAF in the major modified product was at the C(8) position of the guanine moiety. The ^{13}C nmr spectra became indispensible supplement to the ^{1}H nmr spectra. Table I shows the ^{13}C chemical shifts of the low field resonances in d(TACGTA) and AAF modified d(TACGTA) relative to dioxane.

The low field resonances arise mainly from the carbon atoms of the purine and pyrimidine bases and the fluorene moiety. Resonance assignment was accomplished using reference compounds and the general sequence of chemical shifts of the carbon atoms in the bases and fluorene agreed well with the established sequences (11). The main advantage of ^{13}C nmr study is that individual ^{13}C resonances resolve better than the ^{1}H resonances. The main disadvantage is that sensitivity is much poorer for ^{13}C than ^{1}H nmr. The natural abundance of ^{13}C is only 1%. The modification of the solution-phase phosphotriester technique in our laboratory for the synthesis of oligodeoxynucleotides made the preparation of fairly concentrated nmr samples relatively easy. We observed that with a 10 mM single strand concentration of the hexanucleotide 1000 scan gave reliable determination of chemical shift of all the carbon atoms. A relaxation delay time of 4 second was allowed between scans to permit relaxation of the nuclear spins.

Figure 1 shows the HPLC profiles of nuclease P_1 digests of d(TACGTA) before and after modification by AAF at C(8) position of the guanine moiety. The profile A) accounts for the chemical structure of d(TACGTA) by resolving the excised, constituent monomers dCmp, dTmp, dGmp, dAmp and dT in the expected ratio of 1:1:1:2:1. The profile B) shows dCmp, some Tmp, Amp and dT along with two additional peaks at 18 and 21 min. (3:1 by area). The internucleotide linkage on the 3′ side of bulky adducted DNA has been reported to be resistant to attack by nuclease P_1 (7). This feature of nuclease P_1 digestion taken together with only partial release of dTmp in the profile B) suggests that the major peak at 18 min may be the AAF adduct of the dinucleotide dpGpT.

In order to identify the peak at 18 min in the profile B) of Figure 1, the dinucleotide d(pGpT) was synthesized and reacted with N-acetoxy-AAF following the procedure used for the synthesis of the AAF modified hexamer. The modified dimer was isolated by preparative HPLC and characterized by ^{1}H nmr and uv spectroscopy.

Table I. ^{13}C chemical shifts of the low field resonances in d(TACGTA) and AAF modified d(TACGTA) relative to dioxane

Carbon atom	d(TACGTA) 30°C	AAF modified d(TACGTA) 30°C	Δδ[a]
T2	82.15	82.34	0.19
	81.64	81.90	0.26
T4	99.15	99.10	-0.05
	98.85	98.83	-0.02
T5	44.15	44.81	0.30
	44.04	44.55	0.51
T6+	69.90	70.19	0.29
	69.90	69.87	-0.03
TCH_3	-54.92	-55.01	-0.09
	-55.28	-55.43	-0.15
A2	85.92	86.20	0.28
	85.83	85.95	0.12
A4	84.37	84.44+	0.07
	84.21		0.23
A5	51.81	51.76+	-0.05
	51.60		-0.16
A6	88.98	86.87+	-0.31
	88.75		-0.08
A8	72.92	73.03+	0.11
	72.75		0.28
C2	89.75	90.12	0.37
C4	99.64	99.34	-0.30
C5	29.47	29.50	0.03
C6	73.16	73.23	0.07
G2	88.44	87.50	-0.94
G4	84.81	84.71	-0.10
G5	48.42	47.93	-0.49
G6	92.51	92.12	-0.39
G8	69.35	74.73	5.38
AAF 1		56.28	
2		69.87	
3		58.07	
4		54.16	
5		53.40	
6		60.29	
7		60.76	
8		58.28	
9		-27.72	
10		78.42	
11		ND	
12		74.73	
13		76.98	
14		ND	
15		-48.53	

[a]Δδ equals chemical shift of AAF-modified d(TACGTA) minus chemical shift of d(TACGTA).
[+]Resonance overlap judging from intensity.
ND, not detectable.

In addition to the T H-6 resonance of the thymine residue at 7.39 ppm, the downfield region of the ^{1}H nmr of the modified dimer showed a set of resonances from 7.2-7.7 ppm integrating 7 protons from H_1, H_3, H_4, H_5, H_6, H_7 and H_8 of the fluorene moiety. The disappearance of G H-8 resonance at 7.92 ppm after modification supported the modification by AAF at C(8) of the guanine residue. The UV spectrum of d(pGpT) showed a shift of λ_{max} from 265 nm to 272 nm after

modification with an additional shoulder at 304 nm due to the fluorene chromophore. The C8-AAF adduct of dpG was prepared as reported earlier (8). Co-chromotography of the synthetic modified dinucleotide with the nuclease P_1 digest of the AAF modified d(TACGTA) enhanced the peak at 18 min (see profile C in Fig. 1) and thereby confirming that the peak at 18 min in the profile B) was in fact the AAF modified d(pGpT). Co-chromatography of the digest with the AAF modified mononucleotide identified the peak at 21 min as C8-AAF adduct of dpG (results not shown).

The nuclease P_1 excised adducts of AAF modified DNA model were postlabeled with dansyl chloride. Typically 1-2 OD of the modified hexamer was incubated with 10 μl nuclease P_1 (1 mg/ml) at 37° in 100 μl 0.1 M sodium acetate buffer, pH 5 containing 0.2 mM ZnCl. HPLC analysis showed that the digestion was complete in 90 min. The adducts were enriched by HPLC on a Radial-Pak LC cartridge 8MBC18 (10μ, 8 mm i.d., 10 cm) using a linear gradient of 0-50% acetonitrile in 0.1 M ammonium acetate buffer, pH 7. The peaks at 18 and 21 min in the profile B) of Fig. 1 were collected, lyophilized and dansylated following the procedure reported earlier (1). HPLC analysis of the dansylated sample using fluorescence detection (excitation 340 nm, emission 520 nm) detected two peaks at 22.2 and 25.2 min (Fig. 2B). The large peak at 8.5 min is due to the hydrolyzed product from excess dansyl chloride. The two peaks at 22.2 and 25.2 min were identified as postlabeled AAF adducts of d(pGpT) and dpG respectively by co-chromatography with dansyl labeled authentic markers (Fig. 2A and 2C).

Having detected and identified the AAF adducts in the model study by nuclease P_1-mediated fluorescence postlabeling assay, the same overall procedure was used to assay AAF modified calf-thymus DNA. The modified DNA (100-10 μg) was digested with nuclease P_1 following reported procedure (10) and the digest, at the end, was filtered in a centrifugal ultrafree microunit with 10,000 NMWL polysulfone membrane. The filtrate was fractionated by HPLC using the same condition as described in the model study (see Fig. 1) and a fraction was collected from 12 to 24 min which excluded the normal nucleotides. The collected fraction was lyophilized and dansylated. A same size sample from the untreated DNA was also processed simultaneously as a control. HPLC analyses of the postlabeled samples are shown in Fig. 3. The profiles represent analysis from 3 μg of DNA sample. The analysis was done under a linear gradient of 20-100% acetonitrile in 0.1 M ammonium acetate using a highly sensitive gain in the fluorescent detector. As a result, the base line showed a gradual upward drift in accordance with the change in the composition of the eluent. Nevertheless, the top profile in Fig. 3 from the modified DNA showed 6 additional peaks compared to the bottom profile from the control DNA. The peaks 6 and 5 which constitute 36% and 19.8% respectively of the total adducts, were identified as C8-AAF adducts of dpG and dpGpT by co-chromatography with the authentic markers.

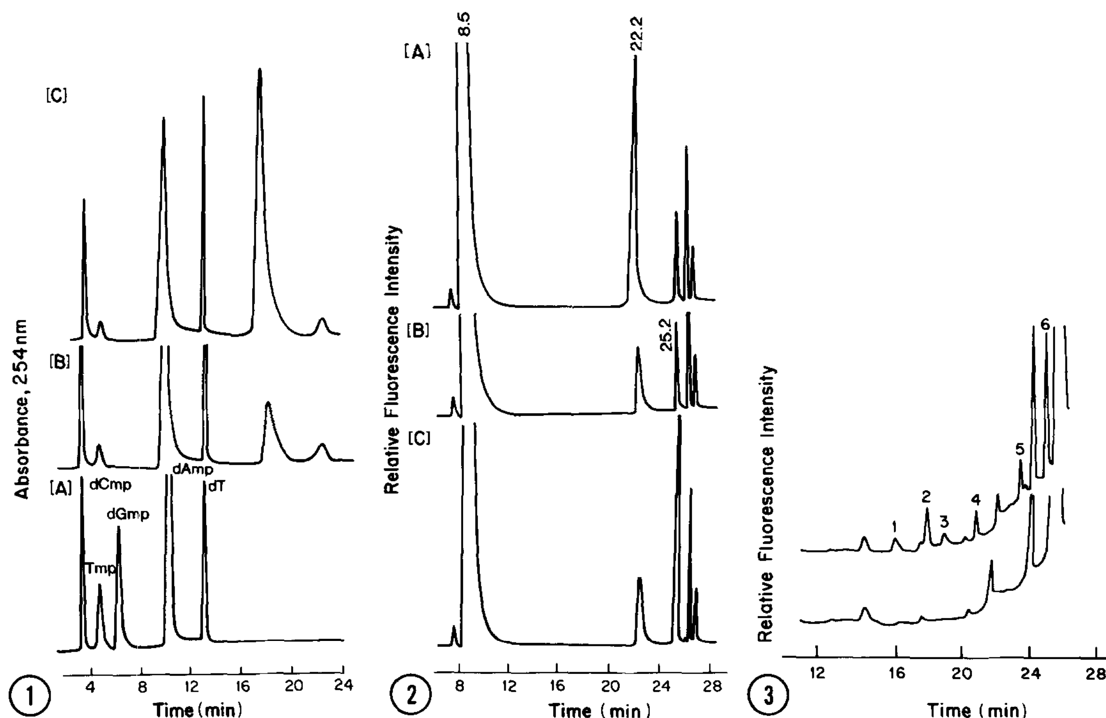

Fig. 1. HPLC elution profiles of nuclease P_1 digests in a Radial-Pak LC cartridge 8MBC18 (10μ, 8 mm i.d., 10 cm) eluted with a linear gradient of 0-50% acetonitrile in 0.01 M ammonium acetate buffer, pH 7. (A) d(TACGTA); (B) AAF modified d(TACGTA); (C) co-chromatography of AAF modified d(pGpT) with the digest of the modified hexamer.

Fig. 2. HPLC elution profiles of dansylated digests in a reversed phase C18 column (5μ, 4.6 mm i.d., 25 cm) eluted with a linear gradient of 20-100% acetonitrile in 0.1 M ammonium acetate buffer, pH 7 and detected with a fluorescent detector with excitation 340 nm and emission 520 nm. (A) AAF modified d(TACGTA) co-chromatographed with dansylated, C8-AAF modified d(pGpT). (B) AAF modified d(TACGTA). (C) AAF modified d(GTACG) co-chromatographed with dansylated C8-AAF modified dpG.

Fig. 3. HPLC elution profiles of dansylated digest of AAF modified calf-thymus DNA (top) and control DNA (bottom). HPLC condition is same as described in Fig. 2.

The residual peaks from 4-1 comprised 7.2%, 7.2%, 19.8% and 9% respectively of the total modification. ^{32}P postlabeling assay of AAF modified calf-thymus 3'-DNA digest detected 56% dG-C8-AAF, 5.8% dG-N2-(C3-AAF), 9.6% and 18.5% two other unidentified adducts along with several minor spots (also unidentified) which amounted to 10.1% of the total modification (9). In a different study, ^{32}P fingerprinting analysis of the distribution of adduct level in Hind III repetitive DNA sequences and total hepatic DNA of rats exposed to the same carcinogen, Gupta et al. (12) discussed the observance of four chromatographically related unknown acetylated derivatives in addition to the

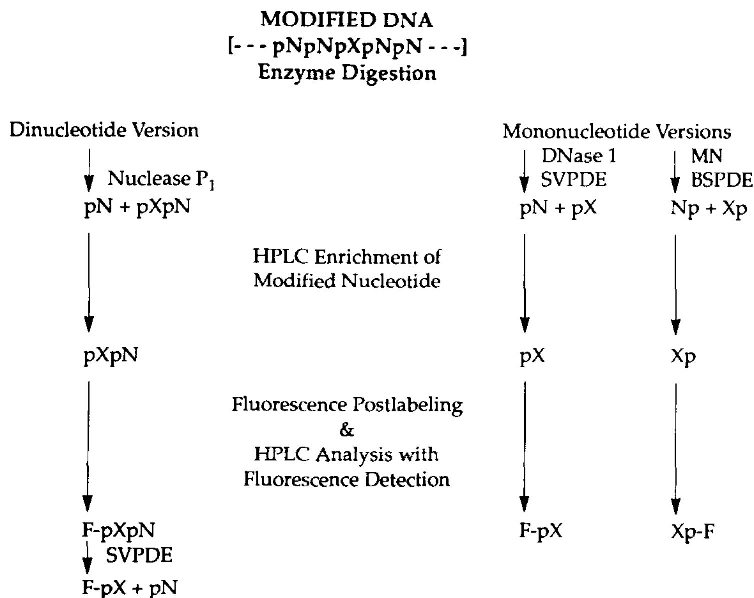

Fig. 4. Scheme of the dinucleotide and the mononucleotide versions of fluorescence postlabeling assay of DNA damage; F (fluorescence label).

known products. These four peaks were later characterized as the undigested dinucleotide of the structure dpXpNp where X is dG-C8-AAF and N is A,T,C or G. The results of our model study taken together with the above report suggest that 3 of the 4 unidentified peaks in the top profile in Fig. 3 seem to result from the structure dpXpN where N is A, C and G. The residual peak is a likely candidate for dG-N2-(C3-AAF) of dpG since this derivative has been reported to be present invariably in both _in vivo_ and _in vitro_ studies of AAF modified DNA (13,14). In the absence of authentic markers it is difficult to assign unequivocally the proper structure for each unidentified peak. However, the present study complements the results of analysis of AAF adducts in calf-thymus DNA by ^{32}P-postlabeling technique (9). Slight variation in the % of the level of the adduct distribution for the various peaks is expected in terms of the different enzymatic pathways used by these two techniques to degrade the modified DNA samples. The detection limit of the analytical system used in this study allows the detection of one modified nucleotide per 10^6 normal nucleotides. The preliminary results of a newly developed analytical system in our laboratory shows, however, the potential for highly sensitive detection of modified nucleotide by fluorescence postlabeling assay (15).

Figure 4 outlines the scheme for the dinucleotide and the mononucleotide versions of fluorescence postlabeling assay. The dinucleotide as well as the mononucleotide versions of the assay share the common features of enzymatic

digestion, HPLC enrichment, fluorescence postlabeling and analysis of the labeled nucleotides with fluorescence detection. The dinucleotide version of the postlabeling assay capitalizes on the resistance of nuclease P_1 to attack the internucleotide linkage on 3′ side of the modified base. Nuclease P_1-mediated fluorescence postlabeling assay of the AAF modified DNA model and calf-thymus DNA demonstrate for the first time, that fluorescence postlabeling technique has the potential to detect modified nucleotide both in the dinucleotide and mononucleotide forms. As a result, the scope of the fluorescence postlabeling assay for DNA damage is widened especially for those modified nucleotides which resist enzymatic excision in the mononucleotide form.

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