Nucleic Acids Research, 1996, Vol. 24, No. 14 **2821–2828**

A viral genome containing an unstable aflatoxin B_1–N7-guanine DNA adduct situated at a unique site

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Received January 26, 1996; Revised and Accepted May 24, 1996

ABSTRACT

A problem that has hindered the study of the biological properties of certain DNA adducts, such as those that form at the N7 atoms of purines, is their extreme chemical lability. Conditions are described for the construction of a single-stranded genome containing the chemically and thermally labile 8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B_1 (AFB_1–N7-Gua) adduct, the major DNA adduct of the potent liver carcinogen aflatoxin B_1 (AFB_1). A 13mer oligonucleotide, d(CCTCTTCGAACTC), was allowed to react with the *exo*-8,9-epoxide of AFB_1 to form an oligonucleotide containing a single AFB_1–N7-Gua (at the underlined guanine). This modified 13mer was 5′-phosphorylated and ligated into a gap in an M13 bacteriophage genome generated by annealing a 53mer uracil-containing scaffold to M13mp7L2 linearized by *Eco*RI. Following ligation, the scaffold was enzymatically removed with uracil DNA glycosylase and exonuclease III. The entire genome construction was complete within 3 h and was carried out at 16°C, pH 6.6, conditions determined to be optimal for AFB_1–N7-Gua stability. Characterization procedures indicated that the AFB_1–N7-Gua genome was ~95% pure with a small (5%) contamination by unmodified genome. This construction scheme should be applicable to other chemically or thermally unstable DNA adducts.

INTRODUCTION

Aflatoxin B_1 (AFB_1) is a fungal metabolite that contaminates the food supply in certain areas of the world. Dietary exposure to AFB_1 is associated with an increased incidence of hepatocellular carcinoma (HCC), especially in populations in which exposure to hepatitis B virus is a frequent event (1). AFB_1 requires metabolic conversion to its electrophilic *exo*-8,9-epoxide (2,3) in order to cause damage to DNA (4). The metabolic activation of AFB_1 is believed to be the first step in the initiation of AFB_1-induced HCC (5). The epoxide reacts with DNA (Fig. 1) to form a population of adducts (6), the principal of which both *in vitro* (3,7–10) and *in vivo* (8,11–13) is 8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B_1 (AFB_1–N7-Gua). The positively charged imidazole ring of this DNA adduct promotes rapid depurination, resulting in apurinic (AP) site formation. Alternatively, under even slightly basic conditions the imidazole ring of AFB_1–N7-Gua opens to form the chemically and biologically stable AFB_1–formamidopyrimidine (AFB_1–FAPY) (1). The initial AFB_1–N7-Gua adduct, AFB_1–FAPY and the AP site, collectively or individually, are the likely chemical precursors to the genetic effects of AFB_1.

Mutations induced by electrophilic forms of AFB_1 have been examined in *Escherichia coli* induced for the SOS response (14–16) and in a number of mammalian systems (17–21). These genetic studies indicate that several mutations, including GC→AT transitions and GC→TA transversions, occur in DNA globally modified with AFB_1. The predominant mutation observed, however, induced or selected *in vivo*, appears to be the GC→TA transversion. There is speculation that the premutagenic lesion responsible for this mutation is the AFB_1-induced AP site (14,22,23), since dAMP is the base most often inserted opposite AP sites in *E.coli* induced for the SOS response (24,25). It has always been a formal possibility, however, that either the original adduct from which the AP site is derived, AFB_1–N7-Gua, or its FAPY derivative could also give rise to this mutation. Unfortunately, the chemical lability of AFB_1–N7-Gua precluded direct evaluation of its genetic effects. Most mutational studies involving the use of singly modified genomes have been carried out with chemically stable DNA adducts (26–28). The procedures used traditionally for the construction of single-stranded singly modified genomes are not well suited to preserve the integrity of many unstable DNA lesions (29). The present work was focused on developing a strategy for the construction of a single-stranded viral genome containing the unstable AFB_1–N7-Gua adduct in a defined position. These studies are the prelude to the evaluation of the comparative mutational properties of AFB_1–N7-Gua and an AP site *in vivo* (30). These procedures should also allow the construction of singly modified genomes containing other chemically or thermally unstable DNA adducts.

MATERIALS AND METHODS

Materials

*Eco*RI was from Boehringer Mannheim. *Hin*fI and *Hae*III were from New England Biolabs. Bacteriophage T4 polynucleotide kinase, T4 DNA ligase and exonuclease III were from Pharmacia.

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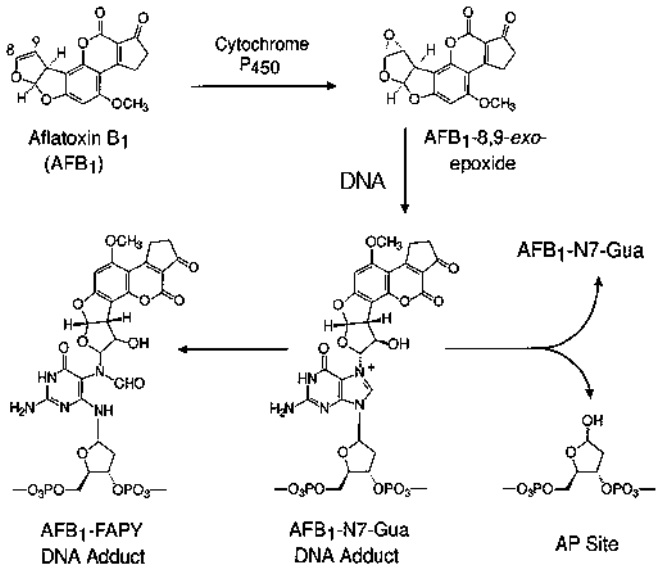

Figure 1. AFB_1 is activated by cytochrome P_{450} to generate its *exo* epoxide (1). The epoxide reacts with DNA to form the primary AFB_1 adduct, AFB_1–N7-Gua, which can undergo depurination to an AP site or opening of the imidazole ring to form AFB_1–FAPY.

Uracil DNA glycosylase (UDG) was from Gibco BRL. [γ-^{32}P]ATP (6000 Ci/mmol) was from New England Nuclear. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Gold Biotechnology. Bacteriophage M13mp7L2 genome was a gift of C. W. Lawrence (31); M13mp7L2 is identical to M13mp7L1 [utilized in experiments described in Banerjee *et al.* (31)] within the polylinker region of the genome. *E.coli* GW5100 (JM103 P1$^-$) was from G. Walker at MIT.

Preparation of AFB_1–N7-Gua oligonucleotides

The *exo* 8,9-epoxide of AFB_1 was prepared as described by Baertschi (32) and the purity was established by ^{1}H NMR. [AFB_1–N7-Gua d(ATGCAT)] (AFB_1–N7-Gua hexamer) and [AFB_1–N7-Gua d(CCTCTTCGAACTC)] (AFB_1–N7-Gua 13mer) were prepared essentially as described by Gopalakrishnan (33) (underlined base indicates position of adduct). Briefly, 60 OD_{260} of oligonucleotide were dissolved in 1.23 ml 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 5×10^{-5} M disodium EDTA. The solution was treated twice with 4 mg AFB_1 epoxide in CH_2Cl_2 solution with vigorous shaking for 10 min at 0°C. The aqueous layer was washed twice with CH_2Cl_2 to remove the AFB_1 dihydrodiol. The AFB_1–FAPY derivative of the hexamer was prepared by treatment of the AFB_1–N7-Gua-modified oligonucleotide with sodium phosphate buffer, pH 9.0, for 4–5 h at 37°C. The solution was adjusted to pH 7, lyophilized and desalted on a Sep-Pak cartridge.

Oligonucleotides were purified by reversed phase HPLC, first on a C-18 semi-preparative column (Alltech Associates) using an acetonitrile/water linear gradient (5–20% CH_3CN in 0.01 M phosphate buffer, pH 7.0, 3.0 ml/min, 20 min) and further on a Beckman Ultrasphere C-18 analytical column using a slightly different acetonitrile/water linear gradient (0–40% CH_3CN in 0.1 M NH_4OAc buffer, pH 6.8, 1 ml/min, 60 min). The column effluent was monitored with a Hewlett Packard 1040A diode array UV-Vis absorbance detector at 260 and 360 nm. The AFB_1-modified oligonucleotide was identified by its characteristic absorbance at 360 nm. The latter conditions were used for all HPLC studies unless otherwise noted.

Stability of AFB_1–N7-Gua

[AFB_1–N7-Gua d(ATGCAT)] was incubated in 50 mM MOPS, pH 6.6, 7.0, 7.4 or 7.8, 50 μg/ml BSA, 10 mM $MgCl_2$ and 20 mM DTT at 16, 25 and 37°C for 30 min and 1 and 24 h. The stability of the hexamer under these conditions was monitored by reversed phase HPLC. The percentages of intact oligonucleotide and any breakdown products were calculated by integrating peaks within the HPLC chromatogram. The percentage of AFB_1–FAPY hexamer was calculated by integrating the peak corresponding to an [AFB_1–FAPY d(ATGCAT)] standard oligonucleotide. The percentage of AP site oligonucleotide was calculated by integrating the peak corresponding to AFB_1–N7-Gua hexamer that had been heated at 80°C, pH 6.6, for 15 min; these conditions efficiently generate the AP site (data not shown).

Characterization of [AFB_1–N7-Gua d(CCTCTTCGAACTC)]

Structural information on the AFB_1–N7-Gua 13mer was provided by electrospray mass spectrometry. In addition, the stability of the AFB_1–N7-Gua 13mer was examined under the exact conditions of genome construction, with the exception that exonuclease III was omitted. The 13mer (1.2 μg) was incubated at 16°C in kinase/ligase buffer (K/L buffer; 50 mM MOPS, pH 6.6, 10 mM $MgCl_2$, 20 mM DTT, 50 μg/ml BSA), in a total volume of 200 μl, in the presence of T4 polynucleotide kinase (39 U) for 15 min. T4 DNA ligase (6 Weiss U) was added and the reaction incubated for 1 h, followed by the addition of UDG (8 U) and incubation for 90 min. The reaction was stopped by the addition of EDTA (20 mM) and stored at –80°C. The stability of AFB_1–N7-Gua under these conditions was monitored by reversed phase HPLC.

Construction of a bacteriophage M13 13 base gapped genome

Single-stranded M13mp7L2 DNA (130 ng/μl) was digested efficiently with *Eco*RI (1.7 U/μl) in 50 mM NaCl, 100 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 100 μg/ml BSA for 2 h at 23°C. The linearized genome was diluted 1.5-fold with H_2O and heated at 80°C for 5 min with a 2-fold molar excess of a 53mer scaffold oligonucleotide (The 53mer scaffold sequence is 5′-AAAAC-GACGGCCAGUGAAUUGAGUUCGAAGAGGCACUGAA-UCAUGGUCAUAGC-3′, synthesized on an ABI PCRmate and gel purified) containing a uracil in place of every thymine and complementary to 20 bases on both the 5′- and 3′-ends of the linearized M13 genome. The mixture was annealed by cooling slowly overnight. This procedure yielded a circular M13 genome containing a 13 base gap complementary to the oligonucleotide d(CCTCTTCGAACTC); the underlined bases are the recognition sequence for *Sfu*I (see Fig. 2). Gapped duplex formation was confirmed by the conversion of single-stranded linear DNA to single-stranded circular DNA as analyzed by agarose gel electrophoresis (1% agarose, no ethidium bromide, gel stained in ethidium bromide subsequent to electrophoresis).

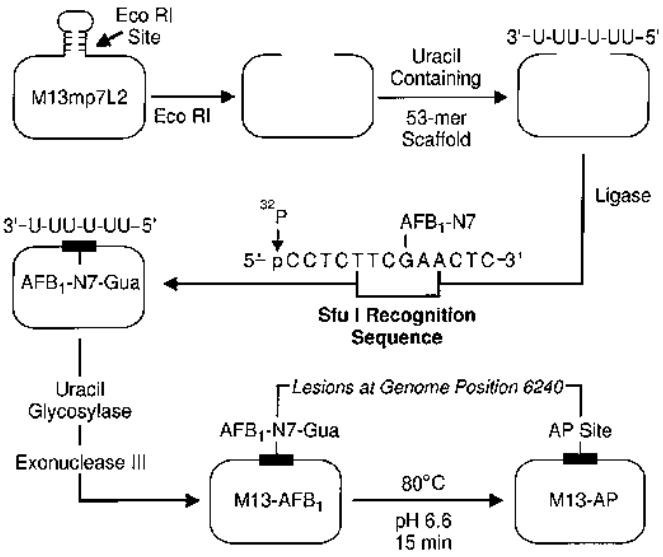

Figure 2. Construction scheme of singly modified single-stranded genomes containing AFB_1–N7-Gua or an AP site. See text for details.

Construction of singly modified genomes

In separate reactions, either unmodified 13mer or AFB_1–N7-Gua 13mer (39 pmol) were ^{32}P 5′-phosphorylated with [γ-^{32}P]ATP by incubation with T4 polynucleotide kinase (10 U) at 16°C for 5 min. The oligonucleotides were ligated for 1 h at 16°C into an equimolar amount of the freshly prepared 13 base gapped molecules (8 pmol/ml) with T4 DNA ligase (0.05 Weiss U/μl). The 53mer uracilated scaffold was removed by further incubation at 16°C for 90 min with UDG (0.04 U/μl) and exonuclease III (0.4 U/μl). (See Fig. 2 for genome construction scheme.) Phosphorylation, ligation and scaffold removal were all done in K/L buffer. A portion of the AFB_1–N7-Gua genome (M13-AFB_1) was heated at 80°C for 15 min in order to release the AFB_1–N7-Gua from the DNA to generate an AP site genome (M13-AP). The unmodified genome (M13-G) was heated in parallel as a control (M13-GΔ). Unincorporated oligonucleotides, ATP, enzymes and salts were removed from the radiolabeled genomes by passing the ligation mixture through a Sepharose CL-4B column (15 × 0.75 cm) pre-equilibrated with 10 mM MOPS, pH 6.6, 100 mM NaCl, 1 mM EDTA. Columns were run at 4°C; the genome eluted in the void volume.

Evaluation of the efficiency of uracil-containing scaffold removal

A new single-stranded M13 genome, designated M13+, was prepared by ligation of unmodified oligonucleotide into the gapped heteroduplex molecule prior to transfection into *E.coli* to generate M13+ phage. Single-stranded M13+ DNA was prepared and annealed to [γ^{32}-P]ATP 5′-phosphorylated uracilated scaffold; the scaffold was present in a 10-fold molar excess to ensure that all M13+ molecules were annealed. The efficiency with which the uracilated scaffold was removed by the combined activities of UDG and exonuclease III was determined by incubating 500 ng scaffold-annealed M13+ in a total volume of 20 μl in the presence of both UDG (0.5 U) and exonuclease III (5 U) for either 90 min or 2 h in K/L buffer. The DNA was then electrophoresed through a 1% agarose gel to separate single-stranded circular and single-stranded linear forms. The efficiency of scaffold removal was determined by measuring the disappearance of ^{32}P. A Molecular Dynamics PhosphorImager was used for this analysis and for all quantification described in the following sections.

Evaluation of the efficiency of removal of AP sites by exonuclease III from the AFB_1–N7-Gua genome preparation

AFB_1–N7-Gua 13mer was ^{32}P 5′-phosphorylated and heated at 80°C at pH 6.6 for 15 min to generate an AP site 13mer that was ligated into the 13 base gapped molecule containing the uracilated scaffold. A portion of the ligation mixture was incubated with UDG and exonuclease III as described in the genome construction section in order to remove the scaffold and inactivate (linearize) the AP site genome. The reaction products were electrophoresed through a 1% agarose gel to separate single-stranded circular and single-stranded linear DNA. The efficiency of AP site genome inactivation was determined by measuring the disappearance of ^{32}P from the circular band on the agarose gel.

Characterization of the AFB_1–N7-Gua and AP site singly modified genomes

The integrity of the AFB_1–N7-Gua adduct within the M13 genome (at base 6240) was determined as follows. A portion of ^{32}P-labeled M13-AFB_1 was heated at 80°C, pH 6.6, for 15 min to generate M13-AP. Subsequently, a portion of the genome was treated with 0.1 M NaOH at 100°C for 10 min to cleave the AP site. These conditions were shown to preserve the integrity of the unmodified and FAPY-containing genomes. A portion of the base-treated DNA was then incubated in 1 M piperidine at 90°C for 1 h to cleave any potentially contaminating AFB_1–FAPY molecules (M13-FAPY) (34). Piperidine treatment was shown to maintain the integrity of the unmodified genome. The DNA was then digested at 37°C for 1 h with single-stranded restriction enzymes *Hin*fI (100 U) and *Hae*III (75 U) in MSB restriction endonuclease buffer (50 mM NaCl, 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT). These enzymes cleave the single-stranded genome at sites flanking the 5′- and 3′-ends of the ligation site. The samples were electrophoresed through a denaturing 20% polyacrylamide gel and the gel was autoradiographed.

Quantitation of M13-G, M13-GΔ, M13-AFB_1 and M13-AP

Exonuclease III treatment, used in the final step of genome construction for removal of the scaffold, was expected to degrade, to a certain extent, the M13 genome in a non-specific fashion. Therefore, it was necessary to determine the final yields of M13-G and M13-AFB_1 after scaffold removal and M13-GΔ and M13-AP after scaffold removal and heating. First, the ligation efficiency of the unmodified 13mer into the 13 base gapped genome was determined. An aliquot of the ligation reaction with unmodified 13mer (without scaffold removal) was heated at 80°C for 15 min to inactivate the ligase. The heated ligation mixture was cooled slowly overnight in MSB restriction endonuclease buffer to ensure that the scaffold, most of which presumably denatured during the heating step, was completely

re-annealed. The genome was subsequently digested with *Hin*fI (40 U) and *Hae*III (50 U) and the entire sample electrophoresed through a 20% denaturing polyacrylamide gel. A known amount of ^{32}P 5′-phosphorylated unmodified 13mer was included on the gel as an internal standard. The percentage of completely ligated material was determined by comparative PhosphorImager analysis of the 31 nt band, which represented completely ligated *Hin*fI/*Hae*III-digested genome, with the internal oligonucleotide standard (data not shown; band sizes are described in Results). Due to the lability of AFB$_1$–N7-Gua, the heating step required for ligase inactivation precluded this analysis for M13-AFB$_1$.

In order to determine the final yield of M13-G, another portion of the unmodified ligation reaction was treated with exonuclease III and UDG to remove the scaffold. Equimolar amounts of the untreated genome and UDG/exonuclease III-treated genome (M13-G) were electrophoresed through a 1% agarose gel to separate single-stranded circular from single-stranded linear DNA. The M13-G yield was determined by comparing the amount of radioactivity present within the circular band of M13-G with the amount of radioactivity present within the circular band of the untreated genome (yield determined above). Electrophoresed on the same gel were equimolar amounts of M13-AFB$_1$, M13-AP and M13-GΔ. The final yields of these genomes were determined by comparison of the amount of radioactivity present within the circular band of each with that present within the circular band of M13-G. Quantitations were done in triplicate.

RESULTS

Strategy for construction of singly modified genomes

A procedure is described for the construction of a biologically active single-stranded M13 genome containing, at genome site 6240, the chemically and thermally labile AFB$_1$–N7-Gua DNA adduct. The work established the optimal conditions for preventing the two principal modes of chemical degradation of the DNA adduct: depurination and opening of the positively charged imidazole ring (Fig. 1).

The ends of a linearized single-stranded M13mp7L2 genome were brought to within 13 nt of one another by a uracil-containing scaffold to yield 13 base gapped genomes (Fig. 2). The gapped genomes were analyzed by agarose gel electrophoresis and a typical yield for gapped genome formation was 50%, with 50% linear M13mp7L2 remaining. Subsequently, the gap was bridged by the 5′-phosphorylated AFB$_1$–N7-Gua 13mer. Removal of the complementary 53mer scaffold by UDG and exonuclease III yielded the single-stranded M13-AFB$_1$ genome. When unmodified 13mer was employed the product was M13-G. Subsequent heating of M13-AFB$_1$ and M13-G at 80°C, pH 6.6 for 15 min yielded M13-AP and M13-GΔ respectively. Electrophoretic analysis of the genomic constructs is shown in Figure 3.

Synthesis, stability and characterization of AFB$_1$–N7-Gua oligonucleotides

In order to determine the conditions most suitable for genome construction it was first necessary to assess the stability of AFB$_1$–N7-Gua in DNA. The AFB$_1$–N7-Gua hexamer was subjected to a range of pHs and temperatures for various amounts of time. The stability of the hexamer was monitored by reversed phase HPLC (elution time ~43 min; data not shown). The relative

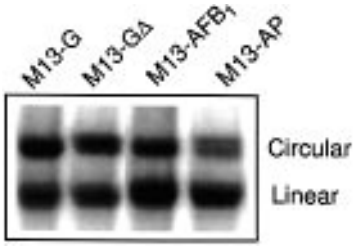

Figure 3. Single-stranded M13mp7L2-derived genome migration on a 1% agarose gel showing both single-stranded circular and single-stranded linear DNA. The 13mer oligonucleotides were ^{32}P 5′-phosphorylated prior to genome construction. The gel was dried and autoradiographed. The circular DNA represents potentially viable genome. Linear DNA should not be viable and is the result of either non-specific exonuclease III degradation of the circular DNA or 13mer oligonucleotide ligation to either the 5′- or 3′-side of the gapped heteroduplex molecule.

amounts of starting material and breakdown product(s) were calculated by integrating chromatographic peaks and the data were graphed accordingly (Fig. 4). These data emphasize that low temperature is critical for AFB$_1$–N7-Gua stability. After incubation for 24 h at 37°C at all four pHs AFB$_1$–N7-Gua was degraded extensively (Fig. 4A–D). Degradation was considerably less after 24 h at 25°C at all four pHs (Fig. 4E–H). In contrast, AFB$_1$–N7-Gua was fairly stable after incubation at 16°C at all four pHs, even after 24 h (Fig. 4I–L). As expected, there was an increase in formation of the AFB$_1$–FAPY derivative (elution time ~40 min; data not shown) as the pH increased. The AFB$_1$–FAPY degradation product was identified by its retention time, identical to the AFB$_1$–FAPY standard oligonucleotide, and also by its characteristic absorbance profile at 360 nm, which is distinct in appearance from the AFB$_1$–N7-Gua profile at 360 nm. The largest percentage of AFB$_1$–FAPY (~50%) was present after 24 h at 37°C, pH 7.8 (Fig. 4D). In contrast, AFB$_1$–FAPY was present at <1% after incubation at pH 6.6 at all three temperatures for 24 h (Fig. 4A, E and I). The AP site oligonucleotide (elution time ~31 min; data not shown) was the major breakdown product at this pH. Since our goal was to optimize conditions for the preparation of as pure an AFB$_1$–N7-Gua genome as possible and the genome construction conditions provided a method for elimination of contaminating AP site, we chose conditions that generated the least amount of AFB$_1$–FAPY while retaining the most AFB$_1$–N7-Gua. The data indicated that the optimal temperature and pH combination for AFB$_1$–N7-Gua stability was 16°C and pH 6.6 (Fig. 4I). After 24 h under these conditions 81% of the AFB$_1$–N7-Gua hexamer remained, no significant level of AFB$_1$–FAPY was generated and 19% AP site was formed. AP site formation was judged tolerable since the final step in genome construction involved treatment with the AP endonuclease exonuclease III. Exonuclease III was shown to be highly efficient in the removal of AP site genomes (see below).

Preliminary studies indicated a very low extent of ligation (~10%) of the AFB$_1$–N7-Gua hexamer, described above, into an M13mp7L2-derived six base gapped genome. To increase the yield of the AFB$_1$–N7-Gua genome a longer singly adducted AFB$_1$–N7-Gua oligonucleotide (13mer) was synthesized. The purity of the AFB$_1$–N7-Gua 13mer was assessed by HPLC (Fig. 5A) and further established by the presence of a single band on a denaturing polyacrylamide gel (data not shown). HPLC data

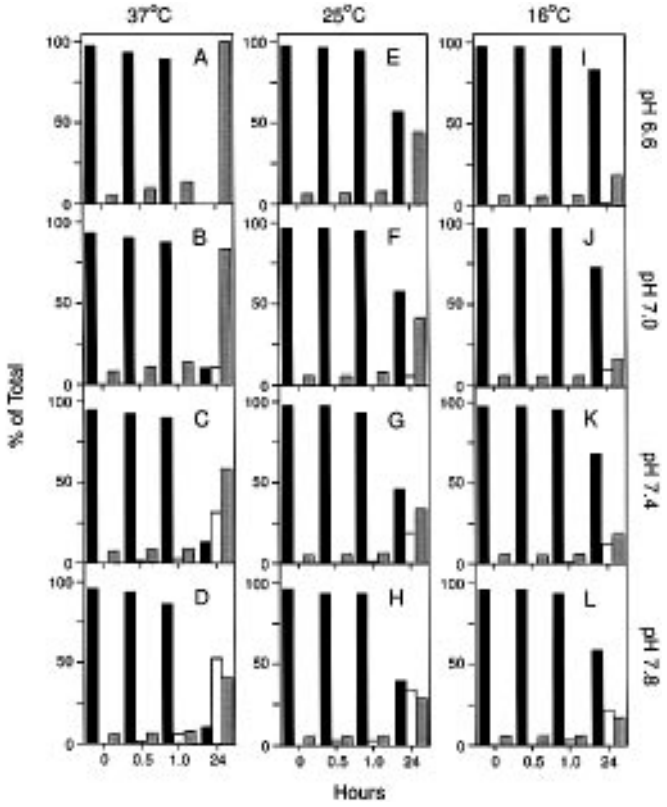

Figure 4. [AFB_1–N7-Gua d(ATGCAT)] was incubated at the indicated temperatures and pHs. AFB_1–N7-Gua stability within the oligonucleotide hexamer was monitored by reversed phase HPLC (see text for details) and the resulting data graphed accordingly. Black bars represent the AFB_1–N7-Gua hexamer, open bars represent the AFB_1–FAPY hexamer and shaded bars represent the AP site hexamer. The conditions determined to be optimal for AFB_1–N7-Gua stability are depicted in (**I**) (pH 6.6, 16°C).

indicated an AP site contamination of 2%. Electrospray mass spectrometry of the monoanion indicated a large peak, eluting at 14.6 min, that consisted of an intense signal at 1390.0 (M-3H)/3z and a weak one at 2084.9 (M-2H)/2z, representing the AFB_1–N7-Gua 13mer; observed molecular weight 4172.4, calculated 4173.8. A much smaller peak preceded the large peak, at 12.3 min, and appeared to consist of a mixture of unmodified and AP site 13mer. Peaks at 1281.5 (M-3H)/3z and 1921.5 (M-2H)/2z likely represent unmodified 13mer (observed molecular weight 3846.6, calculated 3845.6) and peaks at 1236.5 (M-3H)/3z and 1854.5 (M-2H)/2z likely represent AP site 13mer (observed molecular weight 3711.8, calculated 3712.5). It is worth noting that no AFB_1–FAPY oligonucleotide was observed (calculated molecular weight 4191.8).

The stability of the AFB_1–N7-Gua 13mer was examined under the conditions of genome construction (Fig. 5B), except in the absence of exonuclease III, which slightly degraded both control and modified oligonucleotides. Importantly, although the phosphodiester bonds yielded to exonuclease III treatment, the AFB_1–N7-Gua moiety remained intact (data not shown). HPLC analysis revealed that under the conditions of genome construction <1% of the AFB_1–N7-Gua was degraded to an AP site, yielding a total AP site contamination of ~3%. No AFB_1–FAPY oligonucleotide was generated. Evidence that the AFB_1–N7-Gua moiety remained intact within the oligonucleotide was indicated

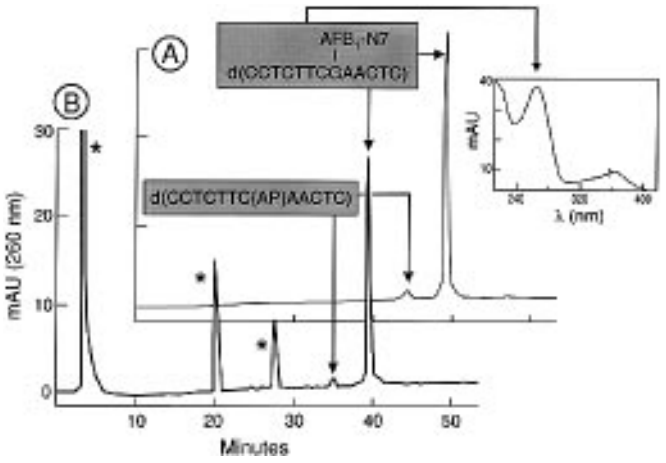

Figure 5. Reversed phase HPLC profiles and UV spectra (inset) of (**A**) [AFB_1–N7-Gua d(CCTCTTCGAACTC)] and (**B**) the same oligonucleotide after incubation under the genome construction conditions (pH 6.6, 16°C, for 165 min). Peaks denoted * represent buffer components as for buffer control (not shown).

by its characteristic chromophore absorbing at 360 nm (inset, Fig. 5). It was thus concluded that the AFB_1–N7-Gua oligonucleotide was adequately stable under the genome construction conditions. Furthermore, these data suggested that essentially pure AFB_1–N7-Gua genome could be constructed, presuming that the small amount of contaminating AP site could be efficiently inactivated in the final step of genome construction upon treatment with exonuclease III.

Removal of the uracil-containing scaffold from M13-AFB_1

In order to observe mutations from the single AFB_1–N7-Gua adduct at a frequency that would provide the most information on the mutagenic process, it was essential that the scaffold (Fig. 2) be completely removed, yielding a single-stranded genome at the adduct site. This step diminishes the possibility of adduct repair and/or strand bias during replication (35). AFB_1–N7-Gua is very labile, making removal of the scaffold a non-trivial task. The problem of scaffold removal was overcome by introducing a uracil in place of every thymine in the scaffold. Using this construct the scaffold could be gently removed by the complementary activities of UDG and exonuclease III. In a model reaction, a ^{32}P 5′-phosphorylated scaffold annealed to single-stranded M13+ DNA was completely removed in 90 min at 16°C, pH 6.6 by the combined activities of UDG and exonuclease III (data not shown). It is worth noting that exonuclease III alone was not sufficient for scaffold removal.

Contaminating AP site genome can be efficiently inactivated by its selective sensitivity to exonuclease III

It was our belief that the AP endonuclease activity of exonuclease III would efficiently inactivate any contaminating AP site genome generated during the synthesis of M13-AFB_1. To test this hypothesis, an AP site 13mer was generated and ligated into the 13 base gapped genome. The ligation mixture was incubated with UDG and exonuclease III to remove the scaffold and linearize the AP site genome. Electrophoresis of the reaction mixtures through a 1% agarose gel indicated that 95% of the material was not only

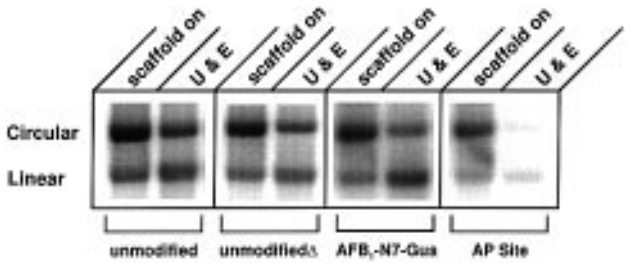

Figure 6. Autoradiogram showing the efficiency of removal of potentially contaminating AP site genome from the M13-AFB_1 preparation. A ^{32}P 5′-phosphorylated AP site 13mer was used to construct an AP site genome containing the uracilated 53mer scaffold. Prepared in parallel were genomes containing an unmodified 13mer, an unmodified heated (Δ) 13mer and an AFB_1–N7-Gua 13mer. Subsequent to ligation of the 13mers, each genome was incubated for 90 min at pH 6.6 (see text for details) with either no enzyme (scaffold on) or with UDG and exonuclease III (U & E). Reaction products were electrophoresed through a 1% agarose gel, the gel was dried and autoradiographed and shows the separation of single-stranded circular and single-stranded linear genomes. Inactivation of the AP site genome by exonuclease III was confirmed by the disappearance of ^{32}P from the circular band after removal of the scaffold.

linearized, but digested so efficiently that the 3′→5′ exonuclease activity removed most of the ^{32}P label (Fig. 6). It was therefore estimated that ~95% of the contaminating AP site genome generated during M13-AFB_1 construction should have been destroyed by exonuclease III. Thus, given a 3% potential AP site contamination, it can be predicted that only 0.15% AP site genome should remain. This extent of AP site contamination was found to be insignificant based on biological studies with M13-AFB_1 and M13-AP (30).

Characterization of singly modified genomes

Oligonucleotide stability and AP site removal studies suggested that <0.2% of M13-AFB_1 was contaminated with AP site genome. Further studies were necessary, however, in order to determine the extent of contamination by unmodified or AFB_1–FAPY genomes.

Genome characterization involved restriction digestion of the genomes with single-stranded restriction enzymes *Hin*fI and *Hae*III, which cleave at sites flanking the 5′- and 3′-ends of the ligated oligonucleotide. As indicated in Figure 7A, *Hin*fI/*Hae*III digests potentially yield a 31 nt fragment representing completely ligated 13mer, a 21 nt fragment representing oligonucleotide ligation only on the 5′-end of the gap and a 23 nt fragment representing oligonucleotide ligation only on the 3′-end of the gap. As indicated in Figure 7B, prior to restriction digestion, the genome was heated to convert the AFB_1–N7-Gua moiety to an AP site that was then cleaved by alkali. This procedure yields a ^{32}P-labeled 15 nt fragment *only* if there were cleavage at the original site of modification. Since unmodified and AFB_1–FAPY genomes were stable under these conditions, as indicated by studies performed on the respective oligonucleotides (data not shown), and we are assuming that <0.2% represents AP site genome, such a band could only represent the original presence of an AFB_1–N7-Gua adduct. The products of each reaction were separated by denaturing PAGE (Fig. 7C). First, the data indicate that heating M13-AFB_1 generated M13-AP, as observed by a shift in the ‘32 nt’ fragment (lane 1), representing completely ligated AFB_1–N7-Gua genome, to a faster migrating 31 nt fragment (lane 2), representing completely ligated AP site genome. We have observed that AFB_1–N7-Gua- and AFB_1–FAPY-containing fragments migrate more slowly than their unmodified or AP site-containing counterparts (unpublished results). The faint smear immediately below the ‘32 nt’ fragment is due to unmodified contamination (discussed below) and probably to a small amount of AFB_1–N7-Gua degradation to the AP site during restriction digestion and electrophoresis. Second, subsequent alkaline treatment resulted in cleavage of the 31 nt AP site DNA fragment, as represented by its conversion to a 15 nt fragment (lane 3). In three separate experiments, ~5% of the 31–‘32’ nt band in lane 1 remained after heat/alkali treatment as determined by PhosphorImager analysis (lane 3). These data suggested that M13-AFB_1 was >95% pure, again assuming an insignificant amount of AP site. In order to determine, however, if the remaining 5% was M13-FAPY as a contaminant, a third step involved piperidine treatment of a portion of the heat/alkali-treated DNA prior to restriction digestion to cleave any AFB_1–FAPY moieties (34). As indicated in lane 4, there was no additional cleavage upon piperidine treatment, indicating the absence of M13-FAPY contamination. Furthermore, since our studies have indicated that the AFB_1–FAPY moiety retards oligonucleotide migration similarly to the AFB_1–N7-Gua moiety, we would expect restriction digested M13-FAPY DNA to migrate similarly to restriction digested M13-AFB_1 DNA, as a ‘32 nt’ fragment. This was not observed; the remaining 5% migrated as a 31 nt fragment and thus was determined to be unmodified contamination. These results, in combination with AP site removal studies, are consistent with the conclusion that M13-AFB_1 was 95% pure with an unmodified contamination of 5%. The unmodified control was unaffected by these conditions (lane 6). See the figure legend for a description of the other bands.

Quantitation of M13-G, M13-GΔ, M13-AFB_1 and M13-AP

The ligation efficiency of the unmodified oligonucleotide into the 13 base gapped genome was 88%. After removal of the scaffold, 52% of the single-stranded circular material remained, giving an overall M13-G yield of 46%. As indicated above, the decrease in DNA was probably due to non-specific exonuclease III degradation. The amounts of M13-GΔ, M13-AFB_1 and M13-AP were determined by comparison of the amount of single-stranded circular material present for each genome, on an agarose gel, with the amount of single-stranded circular M13-G DNA, assuming that the latter was present at 46% yield. The final yields were 38% for M13-GΔ, 25% for M13-AFB_1 and 18% for M13-AP.

DISCUSSION

Evaluation of the genetic effects of DNA lesions is an essential step toward understanding the molecular etiology of chemically induced carcinogenesis. Although the study of the genetic effects of single DNA lesions has been underway for more than a decade, preparation of singly modified genomes containing chemically labile lesions such as AFB_1–N7-Gua for use in genetic studies has been ignored for the most part. The many enzymatic and chemical steps needed to prepare site-specifically modified genomes containing such lesions employ conditions that can alter the structure of these important adducts.

We have developed a procedure with which to construct a single-stranded M13 genome containing the chemically and

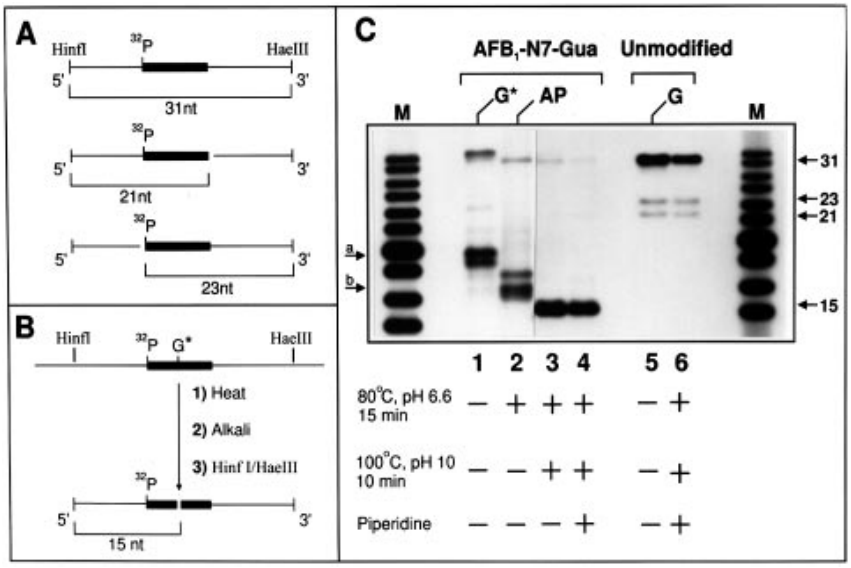

Figure 7. Characterization of M13-AFB_1, M13-AP and M13-G. (**A**) Map of the *Hin*fI/*Hae*III restriction fragments containing the ligated 13mers (black bars). The sizes of completely ligated and partially ligated fragments are depicted. (**B**) Characterization procedure for M13-AFB_1. G*, AFB_1–N7-Gua. (**C**) Autoradiogram of the reaction products electrophoresed on a 20% denaturing polyacrylamide gel. The gel shows radioactive fragments obtained from either M13-AFB_1 (G*) (lane 1), M13-AP (AP) (lane 2) or M13-G (G) (lane 5). Lane 3 is the result of heat/alkaline treatment of M13-AFB_1. Lanes 4 and 6 are after heat/alkali/piperidine treatment of M13-AFB_1 and M13-G respectively. Lanes headed M are 8–32 nt oligonucleotide size markers. Lanes 1 and 2 and 3–6 are from two separate gels. [a]The bands at ~18–19 nt in lane 1 are believed to represent *Hin*fI/*Hae*III-digested genome, ligated at only the 5′-end of the oligonucleotide where the 3′→5′ exonuclease activity of exonuclease III was blocked by the AFB_1–N7-Gua moiety. [b]The bands at ~16–17 nt in lane 2 are believed to represent material resulting from depurination of the material described in [a] (lane 1).

thermally unstable AFB_1–N7-Gua adduct and its AP site counterpart. A 5′-phosphorylated AFB_1–N7-Gua 13mer was ligated into a gap produced by manipulation of the M13mp7L2 bacteriophage genome; the procedure employed is similar to that of Banerjee *et al.* (31), except that the scaffold to which the modified oligonucleotide eventually anneals harbors uracil for each thymine. The scaffold was removed by UDG and exonuclease III to yield single-stranded M13-AFB_1. The entire genome construction was completed in <3 h and was carried out under slightly acidic conditions determined to be suitable for AFB_1–N7-Gua stability. Subsequently, a portion of M13-AFB_1 was heated to generate M13-AP. Our data indicate that M13-AFB_1 was ~95% pure; the only detectable impurities were M13-AP (<0.2%) and M13-G (~5%). Since M13-G should not give rise to targeted mutations and M13-AP contamination is extremely small, these contaminants did not interfere significantly with the use of these genomes for *in vivo* mutational studies (30).

This construction approach yielded genomes containing either AFB_1–N7-Gua or an AP site within the *Sfu*I recognition sequence (5′-TTCGAA-3′). Situation of the lesion in a restriction site provided a method for mutant selection during *in vivo* studies, since mutant DNA was refractory to digestion by *Sfu*I. Another noteworthy feature of the genome construction procedure is that ligation of the 13mer into the 13 base gap restored the *lacZ* reading frame, which is out of frame by +2 in the parental M13mp7L2 genome. Thus, completely ligated material yields blue plaques in the presence of X-Gal and IPTG. The AFB_1–N7-Gua adduct is within an in-frame 5′-GAA-3′ codon (5′-CCTCTTCGAACTC-3′). A G→T transversion within this codon yields an in-frame *ochre* (5′-TAA-3′) that is partially suppressed in *E.coli Sup*B hosts, thus yielding light blue plaques. Consequently, only plaques resulting from targeted G→T transversions are light blue, thus providing facile detection of that mutation type. G→T transversions are the principal mutations observed in AFB_1-treated organisms (14,17,18,21) and account for 75% of the mutations observed for AFB_1–N7-Gua in our parallel *in vivo* investigation (30).

The system described in this work should be applicable for the construction of many other singly modified genomes containing structurally labile DNA adducts. We anticipate that the ability to produce DNA substrates containing uniquely situated unstable DNA adducts will facilitate the investigation of the genetic effects of these lesions in both prokaryotic and eukaryotic systems. This type of analysis is crucial toward understanding, at a mechanistic level, the molecular events involved in the early stages of induction of genetic diseases.

ACKNOWLEDGEMENTS

We thank C. Lawrence for M13mp7L2 and L. Tabibian for technical assistance. M. Wood and D. Treiber are thanked for helpful discussions. This study was supported by grants CA52127 and ES03755 from the National Institutes of Health.

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