J. Biochem. **141,** 675–686 (2007)
doi:10.1093/jb/mvm074

Novel Mutation Assay with High Sensitivity based on Direct Measurement of Genomic DNA Alterations: Comparable Results to the Ames Test

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Received February 19, 2007; accepted February 21, 2007; published online March 23, 2007

Almost all of the methodologies developed to date to assay the potential mutagenicity of chemical substances are based on detection of altered phenotypic traits. The alternative approach of directly screening the whole genome for mutations is not feasible because of the logistics of carrying out mass sequencing of genes. Here we describe a novel and highly sensitive mutation assay, which we term the 'genome profiling-based mutation assay' (GPMA) that directly detects mutations generated in genomic DNA. We used GPMA to detect mutations caused by known mutagens such as AF2 and ethidium bromide even at concentrations of 30 ppb. The number of mutations detected was dependent on the number of generations in culture and the concentrations of the mutagens. Almost complete agreement was observed between GPMA and the Ames test in the discrimination of mutagens (63 out of 64). Owing to the high sensitivity of GPMA, the effects of long-term and low-dose exposures and the influence of chemicals of low solubility can also be screened. Thus, genotype-based GPMA can complement the Ames test, which is the standard technology in this field and is based on phenotypic traits.

Key words: Ames test, genome profiling, high sensitivity, mutagenic reagents, mutation assay.

Abbreviations: EthBr, ethidium bromide; GP, genome profiling; GPMA, genome profiling-based mutation assay; μTGGE, micro temperature gradient gel electrophoresis; PaSS, pattern similarity score; *spiddos*, species identification dots.

Chemical substances are used for a wide variety of purposes. Some chemicals, however, have undesirable characteristics such as mutagenicity or carcinogenicity. Once polluted with such malignant chemicals, our environment will face with a difficult problem to remove them. One of the means used to reduce this hazard is to ensure that all newly synthesized chemicals are subjected to a number of risk assessment tests, including for mutagenicity. At present, the most commonly used test for mutagenicity is the Ames test (*1–3*). This test is based on the principle that bacteria carrying a single point mutation can return to a normal phenotype if a second mutation (back mutation) occurs at the site of the first. Thus, a back mutation in bacteria that are *his*$^-$ allow them to be viable in histidine-free medium. A number of other methods have been developed for the detection of mutagenicity or genotoxicity, for example: the Rec assay (*4*), the comet assay (*5*), the GreenScreen genotoxicity assay (*6*, *7*), the micronucleus test (*8*) and the MutaGen assay (*9*). With the exception of the comet assay, which detects degraded DNA, these assays are based on detection of changes in phenotypic traits in indicator bacteria or cells. Phenotypic traits are often affected by various factors making the interpretation of the action of the mutagen difficult. Theoretically, the most straightforward and unambiguous approach for the detection of mutation is to directly screen genomic DNA. However, although it is now feasible to screen for mutagens using whole genome sequencing, the high cost of this approach renders it impractical. One alternative to use of whole genome sequencing is to read a sufficiently large amount of the genome. However, if the amount of DNA to be sequenced is still very large because of statistical requirements, then this approach too will be unrealistic. As is discussed in this article, this actually seems to be the case. In this study, we examined the utility of genome profiling (GP) for assessing the mutagenicity of chemicals. GP is a well-established method (*10*) for determining the similarity of two genomic DNAs, and has been used in taxonomic studies to assign and classify species (*10–15*) (Supplementary Fig. S1). Recently, a linear relationship was demonstrated between UV dose and DNA changes measured by GP (*15*). In this study, experimental protocols were developed for the detection of mutagens at concentrations of ppb and were proved to be applicable

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to a range of chemicals. Our novel GP-based mutation assay (GPMA) was developed to meet the following methodological challenges: (i) direct monitoring of the effect of mutagens, making the interpretation of the action of mutagens simpler and easier; (ii) providing a different approach for mutation assay, *e.g*. a genotype-based approach that has the potential to reveal new insights into mutagenicity; and (iii) highly sensitive detection of mutagens, enabling measurement of mutagenicity at concentrations of ppb, thereby enabling detection of the effects of long-term exposure to a low concentration of mutagens or to reagents with low solubility. Here we show that GPMA can meet all of these challenges.

Our most important finding was that GPMA produced concordant results to the Ames test, which is based on the phenotype, enabling us to study mutation phenomena from different aspects.

MATERIALS AND METHODS

Chemicals and Medium—Two mutagens were intensively tested in this study: AF2 [2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide, CAS 3688-53-7] and ethidium bromide (EthBr) [3,8-diamino-5-ethyl-6-phenylphenanthridium bromide, CAS 1239-45-8], purchased from Aldrich (USA). Other chemicals were selected from the databases of the National Library of Medicine, Toxicology Data Network (TOXNET; http://toxnet.nlm.nih.gov/) and the Chemical Carcinogenesis Research Information System (CCRIS). The chemicals used were: acridine orange [CAS 65-61-2, CCRIS Recorded Number 736], crystal violet [548-62-9, 2464] and safranin [477-73-6, 1215], purchased from Merck (Germany); caffeine [58-08-2, 1314], guanidine monohydrochloride [50-01-1, 8910], dextran [9004-54-0, 2469] and methyl methanesulphonate [66-27-3, 396], from Sigma-Aldrich (USA); 1,2-dichloroethane [107-06-2, 225], phenol [108-95-2, 504], sodium nitrite [7632-00-0, 559], L-ascorbic acid [50-81-7, 57], carminic acid [1260-17-9, 1397], toluene [108-88-3, 2366], sodium dodecyl sulphate [151-21-3, 6272], dibutyl ether [142-96-1, 6010], tert-butyl alcohol [75-65-0, 4755], 1,4-butanediol [110-63-4, 5984], acetonitrile [75-05-8, 1628], manganese chloride tetrahydrate [13446-34-9, 8932], bromophenol blue [115-39-9, 5487], riboflavin [83-88-5, 1904], methanol [67-56-1, 2301], *N*,*N*,*N*′,*N*′-tetramethylethylenediamine [110-18-9, 4870], pyridine [110-86-1, 2926], 1-butanol [71-36-3, 4321], sulpholane [126-33-0, 2310], zinc sulphate heptahydrate [7446-20-0, 5563], iron (II) sulphate heptahydrate [7782-63-0, 7331], orange G [1936-15-8, 881], acrylamide [79-06-1, 7], 1,4-dioxane [123-91-1, 269], triethylamine [121-44-8, 4881], tert-butylamine [75-64-9, 4758], *N*,*N*-dimethylformamide [68-12-2, 1638], glycerol [56-81-5, 2295], copper (II) sulphate pentahydrate [7758-99-8, 5556], potassium chloride [7447-40-7], monoethanolamine [141-43-5, 6250], 2-propanol [67-63-0, 2308], tetrachloroethylene [127-18-4, 579], imidazole [288-32-4, 3345], ethanol [64-17-5, 945], 1-hexanol [111-27-3, EC 203-852-3] (for 1-hexanol, Ames test data were taken from ECB-ESIS (European chemical Substance Information System; http://ecb.jrc.it)), *N*-butyl chloride [109-69-3, 1389], calcium chloride dihydrate [10043-52-4, 1334], hydroxylamine hydrochloride [5470-11-1, 4323], *N*,*N*′-methylene-bis-acrylamide [110-26-9, 4672], acetone [67-64-1, 5953], tetrahydrofuran [109-99-9, 6276], tributylamine [102-82-9, 4879], triethanolamine [102-71-6, 606], formamide [75-12-7, 6240], acetic acid [64-19-7, 5952], iron (III) chloride [7705-08-0, 2299] and urea [57-13-6, 989], all from Wako (Japan); dimethyl sulphate [77-78-1, 265] from Tokyo Chemical (Japan); auramine O [2465-27-2, 1635] from Chroma Gesellschaft (Germany); nickel sulphate hexahydrate [10101-97-0, 3732], 2-mercaptoethanol [60-24-2, 2097] and methylene blue [61-73-4, 833] from Nakarai (Japan). The chemicals were prepared for use by dissolving in Milli-Q water (Millipore, Japan) at a concentration of 0.01 g/100 ml and then filtering through a nucleopore filter (0.22 μm, Millipore, USA).

The three solutions used to make Davis medium (*16*) were autoclaved separately: 500 ml of Davis A (3.5 g K_2HPO_4, 1.5 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$ and 0.25 g $C_6H_5Na_3O_7$), 5 ml of Davis B (0.2 g $MgSO_4$) and 10 ml of Davis C (2 g glucose). All of the reagents used were the top-grade commercially available.

The following reagents were tested either individually or as a set: L-ascorbic acid (1), carminic acid (2), sodium nitrite (3), acridine orange (4), phenol (5), 1,2-dichloroethane (6), methyl methanesulphonate (7), EthBr (8), AF2 (9), crystal violet (10), safranin (11), auramine O (12) and methylene blue (13). Reagents identified by the Ames test as non-mutagenic were tested collectively (G_1–G_5 and G_2'). These groups contained 10 chemicals, each at a concentration of 300 ppb. Group G_1 consisted of toluene, sodium dodecyl sulphate, dibutyl ether, tert-butyl alcohol, 1,4-butanediol, acetonitrile, manganese chloride tetrahydrate, nickel sulphate hexahydrate, bromophenol blue and riboflavin. Group G_2 consisted of methanol, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, pyridine, 1-propanol, 1-butanol, sulpholane, zinc sulphate heptahydrate, iron (II) sulphate heptahydrate, orange G and caffeine. Group G_3 consisted of acrylamide, guanidine monohydrochloride, 1,4-dioxane, triethylamine, tert-butylamine, *N*,*N*-dimethylformamide, glycerol, copper (II) sulphate pentahydrate, potassium chloride and dextran. Group G_4 consisted of monoethanolamine, 2-mercaptoethanol, 2-propanol, tetrachloroethylene, imidazole, ethanol, 1-hexanol, N-butyl chloride, calcium chloride dihydrate and thiamine hydrochloride. Group G_5 consisted of hydroxylamine hydrochloride, *N*,*N*′-methylene-bis-acrylamide, acetone, tetrahydrofuran, tributylamine, triethanolamine, formamide, acetic acid, iron (III) chloride and urea. Group G_2' consisted of G_2 plus 300 ppb of the mutagen dimethyl sulphate. For more detail, see Supplementary Table S1 for Ames test results.

GPMA Method—A detailed description of the GPMA method is provided in Figs. 1 and 2. The method has four steps: (i) culture of indicator bacteria, (ii) DNA preparation and random PCR, (iii) DNA analysis by micro-temperature gradient gel electrophoresis (μTGGE) and (iv) computer-aided normalization of data. Steps (ii)–(iv) are well-established and, collectively, have been termed as GP (*12*).

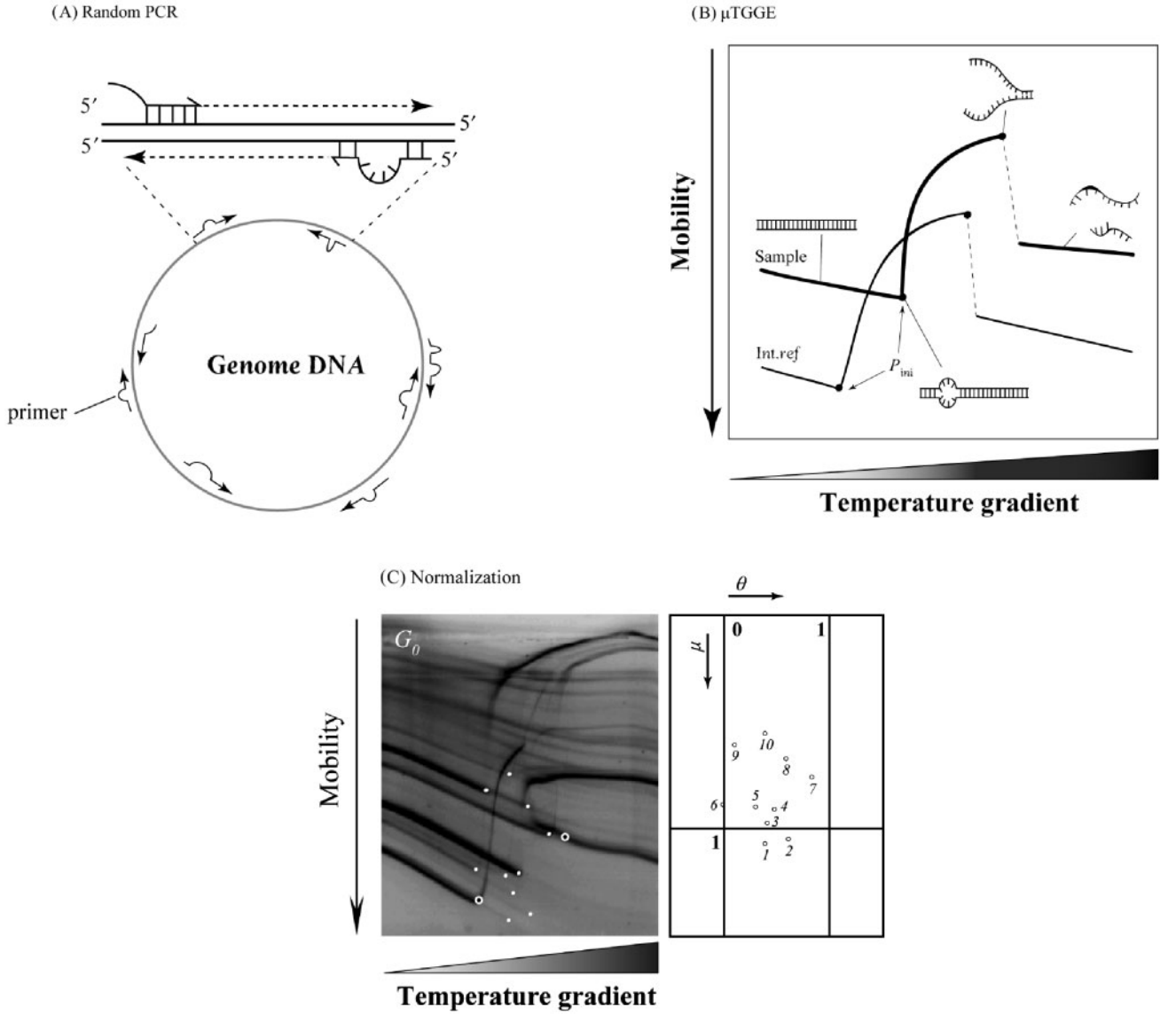

Fig. 1. **A description of the GP method.** GP is composed of three main protocols. (A) Random PCR was carried out using a single (or more) species of primer(s) at lower temperatures than for conventional PCR so as to enable primers to bind with mismatches at various sites on a template. Thus, random PCR enables random sampling of DNA fragments from the whole genome. (B) μTGGE is performed using a slab gel (size of 1 in. × 1 in.) where samples charged on the top of gel migrate in a temperature gradient that is perpendicular to the direction of migration. The point, P_{ini}, which corresponds to the initial melting of DNA in the course of gradual temperature elevation, is processed to generate *spiddos* as shown in Panel C. Schematic drawings of the DNA structures at representative points are shown. The internal reference DNA is used for normalization of the coordinate of a feature point to generate that of *spiddos*. (C) Normalization of a feature point (white dot in the photograph) is carried out using the coordinates of two points (white-fringed black dots) on the internal reference band as reference points. The coordinates of temperature and mobility are expressed by θ and μ after a defined conversion, respectively. Usually, more than five points collected from a single genome profile are used for calculation of PaSS (see text).

Bacterial and Cell Culture—Escherichia coli S26 (Hfr, *met*$^+$, *rel-1*, *lonA-22*, T_2^R, *Su6*$^+$, *phoA4*, PO2A), a derivative of the well-known strain K12, was selected as the standard indicator organism (*17*). Bacteria, stored in 30% (v/v) glycerol at −80°C, were pre-cultured to saturation and then diluted with glucose-free Davis medium (*i.e.* Davis A + B) to (1/200) × concentration. A 2 μl aliquot of the diluted pre-culture was inoculated into a 2 ml tube containing 200 μl of Davis medium. The tube also contained the mutagen under test at a concentration of 1–1000 ppb. It was found that addition of Bovine Serum Albumin (0.1 mg/ml) to this solution was essential in order to prevent the bacteria from adsorbing onto the surface of the vessel and losing their proliferation activity. In each experiment, the level of spontaneous mutation was screened in two simultaneous samples. The bacteria were grown in a closed tube with a reservoir of air (2 ml air for 0.2 ml culture) for 21 h

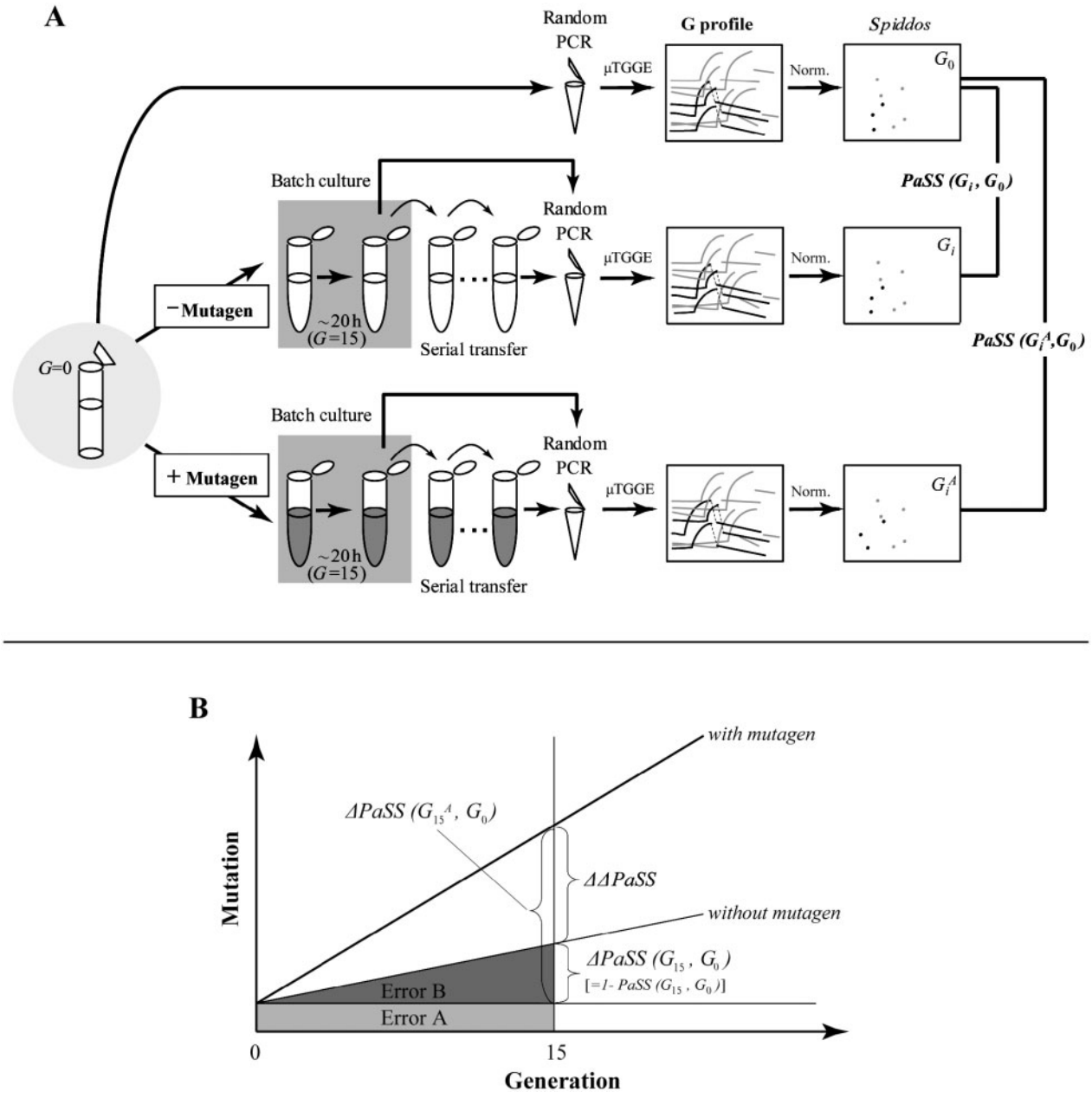

Fig. 2. **Schematic outline of the GPMA.** (A) The indicator bacterium (*E. coli* S26) was cultured in a batch or in a serial transfer mode with or without the mutagen. A sample of the cultured cells was subjected to random PCR and the PCR products were analysed by μTGGE. The resultant band patterns were processed to identify feature points. PaSS was calculated from two sets of *spiddos*, for example G_i (*spiddos* for the culture of *i*-th generation grown in mutagen-free medium)/G_0 (*spiddos* for the original indicator organism before culture), or G_i^A (*spiddos* for the culture of *i*-th generation grown in mutagen A-containing medium)/G_0. (B) Interpretation of the errors observed in GPMA experiments. As the culture of test cells proceeds, the number of mutations increases, depending on the mutagen concentration. Therefore, Error A is a bias equivalent to the gross experimental error (consisting of operational errors and PCR-derived errors), while Error B is associated with the duration of culture (*i.e.* the number of generations and the accumulation of spontaneous mutations) and is obtained as the difference between G_{15} and G_0.

(*i.e.* 15 generations) at 37°C, with shaking. Generation number, *G*, was estimated from the following equation:

$$G = \ln (N/N_0) \ln 2 \quad (1)$$

where N and N_0 represent the numbers of post-culture and starting bacteria, respectively. Where necessary, bacterial turbidity was monitored at 660 nm to measure doubling times. The pre-stationary phase (just after the late-log phase) was selected for the serial transfer experiments. To accumulate mutations, we performed serial transfers to produce multi-generation cultivars. At each transfer, 2 μl of a saturated bacterial culture was

placed in 200 µl of fresh medium containing the same ingredients as described above. In the second and subsequent transfers, freshly grown bacterial cells were used.

Preparation of Genomic DNAs and Random PCR—Cultured cells were used directly as the template for PCR. A 10 µl aliquot was diluted by 10 times with Davis A medium; a 2.5 µl aliquot of this solution was used as the template (~10^4 cells) in a 50 µl PCR mixture. Therefore, for the sake of convenience, we define 'genomic DNA' as the whole DNA, including all satellite and organellar DNAs (*10*, *11*). Random PCR was performed using a single fluorescent primer (*e.g.* a *Cy3*-labelled dodecanucleotide such as pfM12 dAGAACGCGCCTG) at a low annealing temperature (*e.g.* 28°C) (*10*). The PCR solution (50 µl) contained 200 µM dNTP (N = G, A, T and C), 0.5 µM primer, 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 1 unit of *Taq* DNA polymerase (Biotech International, USA) and 2.5 µl of template DNA (approximately 10^4 cells). Random PCR was carried out using cycles of denaturation at 94°C for 30 s, annealing at 28°C for 2 min and extension at 47°C for 2 min using either a TGradient Thermocycler (Biometra, UK), PTC-200 (MJ Research, Massachusetts, USA) or a Thermal cycler (Takara, Japan). PCR products were ethanol-precipitated and then combined with 1 µl of internal reference DNA (IntRef 1), 1 µl of Tris buffer consisting of 40 mM Tris–HCl (pH 8.0), 20 mM sodium acetate, 1 mM EDTA, 0.3% (w/v) phenol red, 0.3% (w/v) xylene cyanol ff and 20% (w/v) sucrose; 3 µl of H_2O was added to dissolve all of the components. IntRef 1, the internal reference DNA was prepared by PCR by amplification of the 1341–1540 nt region of the phage fd gene VIII. The fluorescence labelled primers MA1′ (5′-Cy3 dTGCTACGTCTCTTCCGATGCTGTCTTTCGCT) and MA2′ (dTTGAATTCTATCGGTTTATCA) were used; they generated a 200 bp PCR product with substitutions of a few bases. A conventional PCR program was used: 30 cycles at 94°C for 30 s, 63°C for 1 min and 72°C for 30 s.

µTGGE—PCR products were subjected to µTGGE, a miniaturized version of TGGE using a gel size of 2.5 cm × 2.5 cm × 0.1 cm (*18*). The PCR products were layered on top of a slab gel [6% (w/v) acrylamide gel containing 8 M urea] and then briefly electrophoresed in order to induce the DNAs migrate into the gel. The gel was then placed on the stage of a µTGGE apparatus (Micro TG, Taitec, Japan). The temperature gradient (30–70°C) was set perpendicular to the direction of DNA migration. Electrophoresis was usually performed for 7.5 min at 100 V. Bands were identified by scanning the gel using a fluoroimager (Molecular imager FX, BIO-RAD, USA) with filters for excitation (550 nm) and emission (565 nm) (Supplementary Fig. S2).

Computer-aided Normalization—A set of feature points (5–10) were assigned for each genome profile shown on computer. These points were processed to obtain the normalized mobility and temperature characteristics, termed here *spiddos* (species identification dots) (*12*) (see Figs. 1 and 3). A measure of similarity between two genomes, the pattern similarity score (PaSS), was devised and is defined as follows (*12*):

$$\mathrm{PaSS} = 1 - \frac{1}{n}\sum_{i=1}^{n}\frac{\left|\boldsymbol{P}_i^{G_1} - \boldsymbol{P}_i^{G_0}\right|}{\left|\boldsymbol{P}_i^{G_0}\right| + \left|\boldsymbol{P}_i^{G_1}\right|} \qquad (0 \le \mathrm{PaSS} \le 1) \qquad (2)$$

In this measure, coordinate differences between corresponding *spiddos* are accumulated and subtracted from unity. *Spiddos* are especially important in that the coordinate of each is a unique and exact representation of the corresponding sequence derived from the normalization process of the internal reference (*12*). This process ensures elimination of experimental variables such as instrument noise, inconsistencies in gel formation, temperature differences and so on. Each sequence has a unique *spiddos* at a coordinate derived from two dimensions, *mobility* and *temperature*, which depend on the sequences of both the primer and the template. A point mutation will cause a displacement of a *spiddos* upward or downward, usually by 0.1–0.3°C, towards the temperature axis. Insertion and deletion mutations alter the size of the DNA and change its mobility. The contribution of these mutations can be evaluated using Equation 2, which was formulated using both theoretical and empirical information (*12*). A number of GP experiments have been performed so far, involving more than 200 species of enterobacteria, fungi, protozoa, plants and animals, and have demonstrated the utility and effectiveness of *spiddos* (and PaSS) for identifying species and for measuring interspecies genome distances (*12–14*). Empirically, it is known that PaSS has an error of ≤1.0% if performed by a well-trained investigator (*12*).

Sequencing—Some PCR products were cloned using a TOPO TA cloning kit (Invitrogen, USA) and sequenced either using a DSQ-2000L DNA sequencer (Shimadzu, Japan) or by outsourcing. The following DNA fragments were sequenced: α (265 bp, DDBJ:U00096, ECGS nos. 2,090,923–2,091,165); β (327 bp, DDBJ:U00096, ECGS nos. 1,084,058–1,084,362) and γ (336 bp, DDBJ:U00096, ECGS nos. 1,366,494–1,366,807) where ECGS no. stands for *E. coli* genome sequence number registered by sequencing the genome sequence of *E. coli* K12 (*19*, *20*). Note that the sequence obtained here from S26 had a few substitutions (T → C, A → G); for details see Supplementary Fig. S3.

RESULTS AND DISCUSSION

The basic technology of GP is shown in Fig. 1. A description of the experimental procedures developed here are shown in Fig. 2 together with the interpretation of the errors involved. The results of a typical GP analysis of cells are shown in Fig. 3; in this instance, the GPs of cells before and after culture are compared. Obviously, the feature points (white dots in the photos of Fig. 3) can be easily assigned since they appear at similar coordinates. It should be noted that random PCR products (DNA) are generated in specific amounts

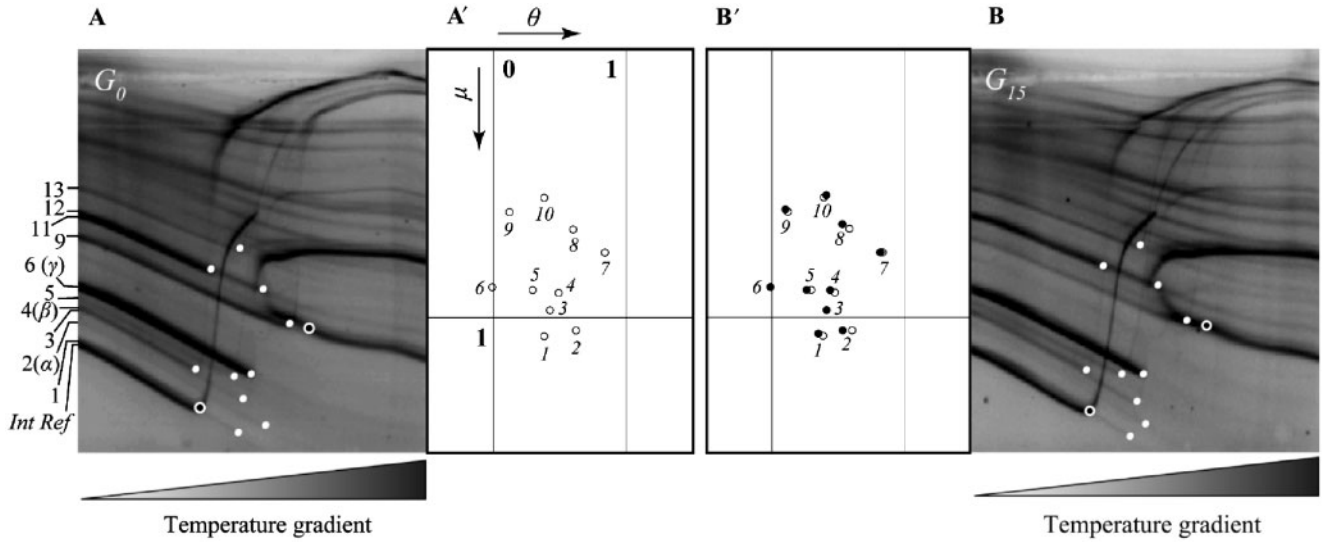

Fig. 3. **GP and *spiddos*.** Raw and processed GP data are shown for pre- and post-cultured bacteria (A (A′) and B (B′), respectively). The DNA bands corresponding to the internal reference (IntRef), α, β and γ bands are indicated in Panel A. *Spiddos* obtained after normalization are plotted on a chart where the axes show normalized mobility (μ) and temperature (θ). (Panels A′ and B′). In Panel B′ *spiddos* obtained for 0 generation bacteria were superimposed (blank circles) over the *spiddos* for 15 generation bacteria (filled circles).

depending on the binding affinity of the primer and template DNA (*20*, *21*). Therefore, this process, in which DNA bands of higher intensities, say top 10, are selected, has a meaning of eliminating such intensity information of each DNA, which might be useful for other purposes, for the sake of simplicity. The raw coordinates were normalized using the coordinate of the internal reference to produce the coordinates of the *spiddos* (*12*) (Fig. 3). Next, the *PaSS* was calculated with Equation 2, using positional differences between corresponding dots: a score of 1 represents a complete match. Most scores were close to but less than 1 (*12*) (Supplementary Fig. S4).

As this technology is novel, we first performed experiments to confirm its reproducibility and effectiveness using the mutagens AF2 and EthBr (Supplementary Fig. S5).

Concentration Effect—The effect of differences in concentration was examined using AF2 as the test mutagen. At concentrations of AF2 up to 200 ppb, there was a roughly linear relationship between the concentration and the degree of mutation measured by GPMA (ΔPaSS) (Fig. 4). This suggests that the rate of mutation increased with concentration of mutagen. However, at concentrations >200 ppb, ΔPaSS fell sharply. At these concentrations, growth experiments showed that the bactericide effect of the chemical became prominent (Fig. 4A). The fall in the rate of mutations may therefore be the result of the survival of strains with fewer mutations and of the elimination of AF2-sensitive and highly mutated cells. This is a noteworthy point when assessing the mutagenicity of chemicals. Our data indicate that a concentration as low as 10 ppb of AF2 is still mutagenic and can be detected by GPMA.

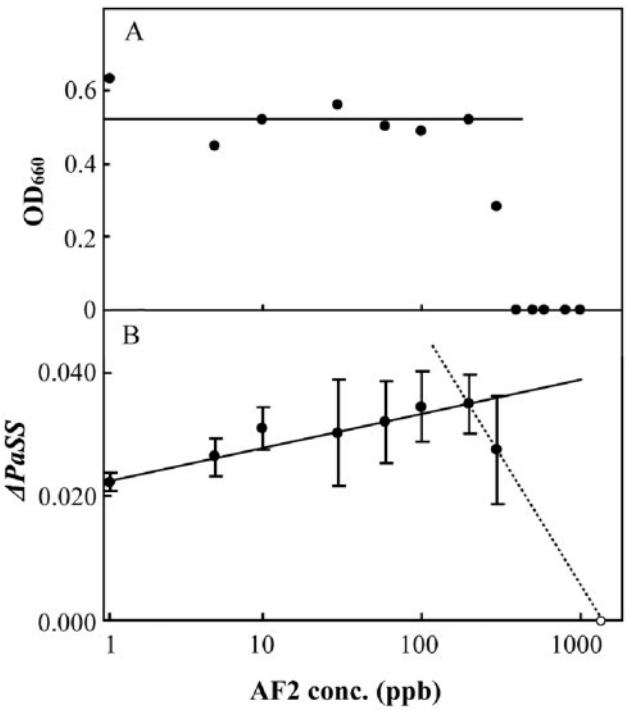

Fig. 4. **Concentration effect of mutagen AF2.** The indicator bacterium, *E. coli* (S26), was cultured at each concentration of AF2 for 21 h (15 generations) at 37°C and monitored at OD_{660} (Panel A: only the average is plotted.). For each concentration, two independent experiments were performed. Triplicate samples were used in each independent experiment. At a concentration of 1000 ppb of AF2, no growth of *E. coli* was observed and is thus marked at the zero level (circle) in Panel B.

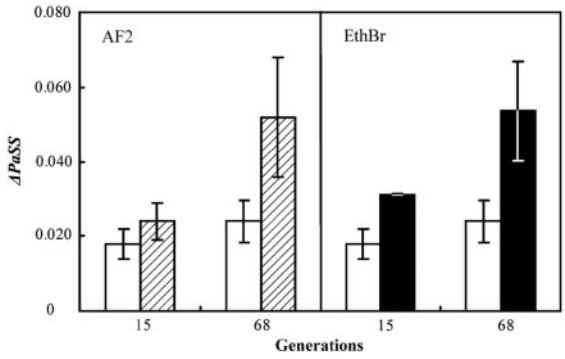

Fig. 5. **Generation-dependent accumulation of mutations.** Bacteria were cultured in 10 ppb of AF2 (hatched) or EthBr (filled); culture conditions were otherwise standard. Open columns are bacteria cultured without mutagens. Experiments were performed in duplicate.

As mentioned above, a UV irradiation experiment provided an estimate for the background error level of ~0.01 (*15*) and also showed that GPMA can quantitatively monitor the accumulation of DNA lesions and mutations. In a separate experiment, DNAs were amplified by random PCR using *Taq* DNA polymerase in the presence of carried-in AF2 (equivalent to culture in 1000 ppb of the chemical) (data not shown). The ΔPaSS results did not differ significantly between experiments with and without AF2. Therefore, the ΔPaSS values obtained from bacteria grown in the presence of AF2 were mainly the result of mutations that had been fixed in the population through the proliferation process.

Effect of Long-term Culture—In order to determine the effects of long-term culture in the presence of a mutagen (10 ppb), we compared populations cultured for either 15 generations (short term) or 68 generations (long term) in the presence of EthBr or AF2 (Fig. 5). For both chemicals, long-term culture resulted in the accumulation of more alterations. A similar outcome was found after comparing bacteria cultured for either 15 or 102 generations in the presence of 10 ppb EthBr (data not shown). Bacteria grown in mutagen-free culture had low ΔPaSS values (~0.02 after 15 generations and ~0.025 after 68 generations); these values were significantly greater than the background error level (<0.01). Overall, our experimental results support the idea that mutations accumulate at a near constant rate in genomic DNAs and that the rate of accumulation depends on the type and the concentration of mutagen. The GP method provides a means for semiquantitative measurement of the rate of accumulation of mutations. Interestingly, the ΔPaSS values obtained from bacteria grown in mutagen-free media in both the AF2 and EthBr experiments increased by ~0.005 between the short- and long-term sampling points (*i.e.* 53 generations). Although these values are comparable to the error ranges, they suggest the level of spontaneous mutation. These experiments clearly show that GPMA really can monitor the accumulation of mutations.

Screening for Mutagenicity using GPMA—Having established the practicality of the GPMA approach, we next screened 64 selected chemicals that have previously been examined by the Ames test (see Fig. 6, Panel A, and Supplementary Table S1 and Fig. S6). We evaluated the mutagenic strength of chemicals used here and examined by the Ames test as shown in Table 1. We found good agreement between the results of GPMA and the Ames test (Fig. 6, B and C). All of the chemicals that were classified by the Ames test as non-mutagenic (including L-ascorbic acid, carminic acid and others) were also found to be non-mutagenic in either individual (Fig. 6B) or group GPMA tests (Fig. 6C). Likewise, both tests showed good agreement in their evaluation of the strength of mutagenicity of the chemicals (Fig. 6, Panels B, C and E). A clear dose-effect was observed for 1,2-dichloroethane (Fig. 6, chemical '6' on Panel B) and methyl methanesulfonate (Fig. 6, chemical '7' on Panel B): both showed no significant mutagenicity at 30 ppb but were mutagenic at 1000 ppb. This experiment also showed that AF2 (Fig. 6, chemical '9' on Panel B) was mutagenic even at 30 ppb (this experiment was performed independently of that shown in Fig. 5) and also highly bacteriotoxic; these characteristics are now well-known and thus this chemical has been banned for use as a food preservative.

The experiment showed that groups of chemicals can be evaluated for mutagenicity; representative results for 10 species of chemicals collectively tested at a concentration of 300 ppb each are shown in Fig. 6C. Clearly, collective evaluation has the potential to save a considerable amount of labour. However, a mutagenicity-positive result does require further analyses to determine which chemicals are responsible. In the G_2' group, which contained 300 ppb dimethyl sulphate in addition to the non-mutagenic chemical of group G_2, the presence of the single mutagenic chemical yielded the expected positive result. We believe that 300 ppb is an appropriate and convenient concentration for general use in GPMA testing; this concentration is lower than those usually employed in Ames tests (1–1000 ppm). It also has the advantages of enabling evaluation of chemicals with low solubility and yet successfully discriminating between all mutagens and non-mutagens tested (Fig. 6C). Undoubtedly, testing concentrations other than 300 ppb may prove of value for confirmation of an initial screen and for a more full characterization of a compound.

There was a remarkably strong agreement between the results obtained from the two assays (Fig. 6E). Thus, both approaches can successfully achieve the same goal of identifying mutagenic agents. The Ames test is indirect as it depends on cell survival following a beneficial back mutation, an outcome that involves many intracellular events. In contrast, the GPMA approach directly measures phenomena occurring in genomic DNA. This property will be of value for detailed characterization of the mutagenicity of chemicals.

Comparison of GPMA and Ames Test—The Ames test is a well-established and highly reliable method for assaying chemical mutagenicity, and a large amount of data has been accumulated using this test since the original report in 1973. In contrast, GPMA is a new technology. Both methods make use of an indicator

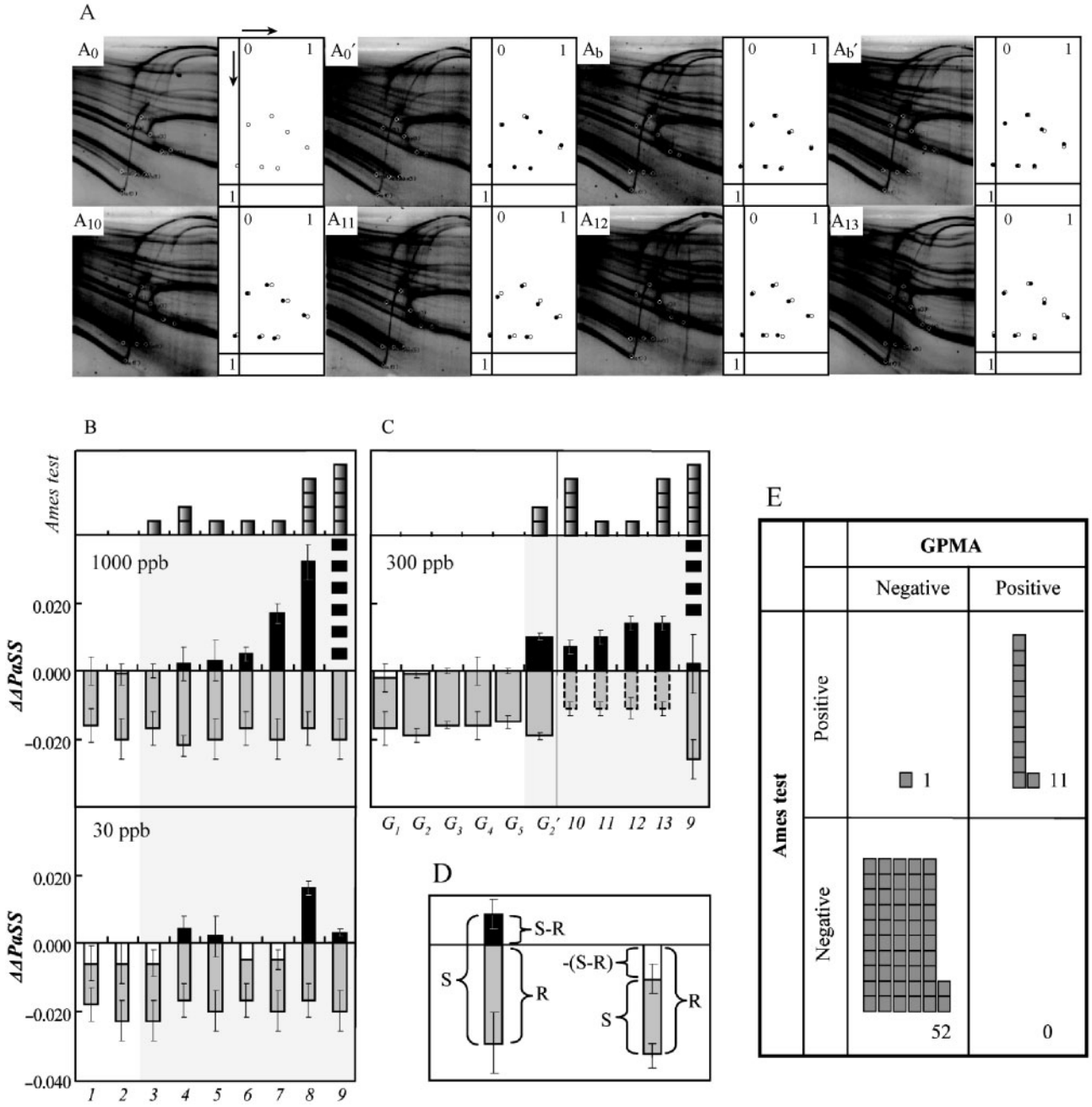

Fig. 6. **Mutagenic and non-mutagenic reagents tested by GPMA.** Sixty-four chemicals for which Ames test data are available were assayed by GPMA. The strengths of these mutagens were evaluated using Ames test data from TOXNET and ECB-ESIS to determine the lowest concentration of each reagent that yielded a positive effect in the Ames test (see also Supplementary Table S1). Panel A shows representative raw data. Each featuring point is shown to be numbered. Blank circles (*spiddos* for A_0) are superimposed throughout. The effect of each reagent was tested at 30 ppb (B, bottom), 1000 ppb (B, middle), and 300 ppb (C) and the results are presented in the order of mutagenic strength at 1000 ppb. G_2' contains the same 10 chemicals with G_2 and dimethyl sulphate in addition. Broken bars indicate 'lethal' or 'partially lethal'. The reagents tested are listed in the 'MATERIALS AND METHODS'. The strength of mutagenicity of each chemical/group was evaluated by ΔΔPaSS, *i.e.* $(\Delta PaSS^S - \Delta PaSS^R)$ where $\Delta PaSS^S$ and $\Delta PaSS^R$ represent the ΔPaSS of sample chemical S and Reference R (no chemical added), respectively. Panel E summarizes the results obtained here. Sodium nitrite was the single exceptional reagent that was Ames test-positive but GPMA-negative.

organism (bacterium), although GPMA does not need a special strain such as the *Salmonella his*$^-$ strain required by the Ames test. Maintenance of a particular strain of bacterium entails considerable effort. Moreover, continuous culture of a strain inevitably leads to the accumulation of mutations, as has been observed in this study (Fig. 5). Therefore, the fact that GPMA does not depend on a particular strain of indicator organism is a

Table 1. **Relative mutagenic strengths (determined by the Ames test) of the reagents used here.**

Reagents		D_{min}[a] (μg)	Mutagenic strength (S)[b]	Strength level (L)[c]	References
No.	Name				
1	L-Ascorbic acid	100	0	0	(*22*)
2	Carminic acid	125	0	0	(*23*)
3	Sodium nitrite	100	0.25	1	(*24*)
4	Acridine orange	20	1.25	2	(*25*)
5	Phenol	–[d]	–[d]	0–1	(*26*)
6	1,2-Dichloroethane	100	0.25	1	(*24*)
7	Methyl methanesulphonate	110	0.227	1	(*27*)
8	Ethidium bromide	0.05	500	4	(*28*)
9	Furylfuramide (AF2)	0.02	1250	5	(*25*)
10	Crystal violet	0.1	250	4	(*28*)
11	Safranin	50	0.5	1	(*29*)
12	Auramine O	33	0.75	1	(*24*)
13	Methylene blue	0.03	833.3	4	(*30*)
$-G_2'$	Dimethyl sulphate	13.3	1.88	2	(*31*)

[a]Minimum effective dose, *i.e.* the minimum amount of reagents per plate (~25 ml) that induced an observable number of revertants (usually ~100/plate).
[b]By definition, 25 ml/D_{min}.
[c]If S is ≤1, ≥1 and <10, ≥10 and <100, ≥100 and <1000 or ≥10^3 and <10^4, then, $L = 1$, 2, 3, 4 or 5, respectively. The height of the columns for Ames tests shown in Figs 6 and 7 corresponds to the value of L.
[d]Below the detection level.

great benefit and allows flexibility in the selection of the indicator organism. There is no reason why GPMA could not be performed using mouse or human cell lines.

The Ames test has been refined using specifically developed strains of *Salmonella* to discriminate between different types of mutation: transition, transversion or frame-shift mutations (*1*, *32–34*). In comparison, GPMA can detect, to some extent, point mutations and insertions/deletions occurring within the DNA fragments used for sampling of *spiddos*, as these mutations cause a vertical or horizontal (or both) shift of *spiddos* (see Figs. 6A, 3B, and 8, Supplementary Fig. S6). The greatest difference between the Ames test and GPMA is a methodological one: one assay is phenotype-based, the other genome-based. That is, the Ames test monitors whether cells have recovered the ability to synthesize histidine to a sufficient level to ensure cell viability. The overall frequency of mutation must be sufficiently high so as to render a detectable event as only a fraction of back mutations will result in the proliferation of cells and the eventual formation of colonies due to the stochastic nature of multistep events required for the proliferation. This requirement means that it is not feasible to screen low concentrations of compounds using the Ames test. As GPMA is free from this constraint, it can be operated at concentrations as low as 10 ppb. GPMA is therefore able to test compounds with low solubility, an aspect of mutagenicity testing that has largely been left untouched. This is a strong advantage of GPMA over the Ames test. However, we also need to bear in mind that certain factors that influence mutation, such as mutator genes, can cause a change in the mutation rate (higher by orders of magnitude) leading to a false-positive conclusion. This phenomenon is also common to the Ames test and other methodologies that are based on bacterial mutation. Fortunately, this phenomenon seems either to be very rare or to be suppressed in GPMA by some unknown mechanism since we have not encountered this problem in our 6 years of experience, judging from the reproducibility of the test. If this phenomenon occurred, then one would anticipate different results from a pair of independent experiments.

Comparison of GPMA with the Sequencing Approach—In order to confirm the rate of mutations in genomic DNA, we cloned and sequenced the same DNA fragments for each sample (~10 kb in total) as those that appear in GP (DNA bands α, β and γ in Fig. 3). The sequencing results are summarized in Fig. 7 (see Supplementary Fig. S3 for detail). On the basis of this sequencing, AF2 was found to be positively mutagenic but EthBr was negative. Since EthBr has already been established by the Ames test as a mutagen that causes mutation by intercalating between the base-stacking of the DNA, the result obtained from the sequencing exercise is clearly unreliable. We used DNAs amplified by PCR in these sequencing experiments; therefore, the sequencing results will inevitably be biased by the mutation rate of the *Taq* polymerase (~10^{-4} per base per replication (*35*)). Although we do not yet have accurate estimates of the mutation rates caused by the chemicals tested, the data obtained here suggest that they must be comparable to or less than that caused by the *Taq* polymerase during GPMA, which includes random PCR in its methodology. Therefore, the apparent inconsistency between the outcomes of the Ames test and the sequencing experiment with regard to EthBr might be a statistical effect resulting from sequencing of an insufficient amount of DNA. In other words, we might have to sequence a much larger amount of DNA to obtain reliable results and to quantitatively measure mutation rates. It must be kept in mind that we cannot detect a lower rate of mutation (signal) than that of the noise level unless the signal-to-noise ratio is sufficiently elevated by performing a large number of experiments (N runs of the same experiment improve the signal-to-noise ratio by

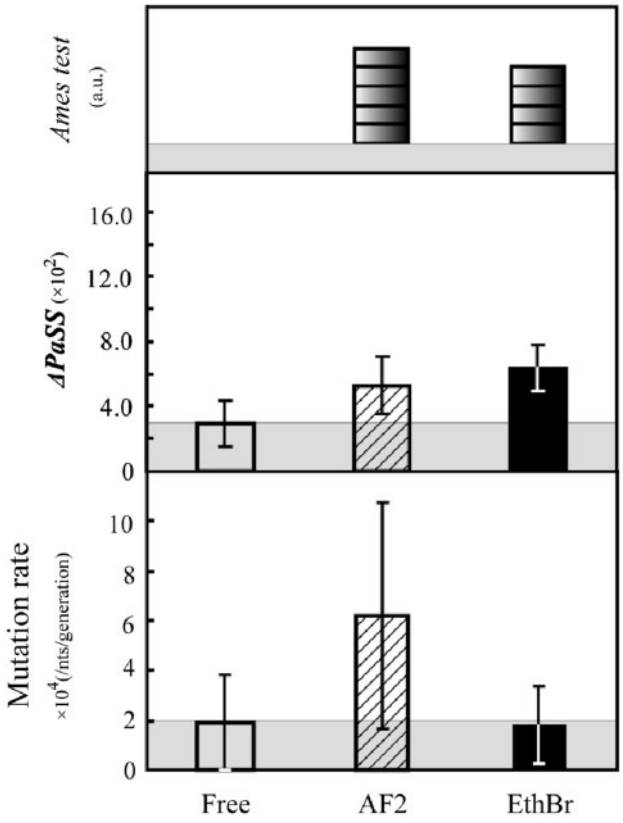

Fig. 7. **Mutagen-dependent mutation rates measured by sequencing.** The same samples as used for GPMA (Trial #1 in Supplementary Fig. S4; that of 30 ppb and 15 generations) were sequenced: bands, α, β and γ shown in Fig. 3. The mutation rates measured for free (no mutagen), AF2 and EthBr were derived from 11 sequences (3252 nts in total), 9 sequences (2784 nts) and 11 sequences (3385 nts), respectively (see also Supplementary Fig. S3). ΔPaSS and the Ames test were basically the same as shown in Fig. 6. Spontaneous mutation levels are shown as the grey zone.

$N/\sqrt{N}(=\sqrt{N})$). Thus, establishment of a sequencing-based mutation assay needs to take these factors into consideration. Intriguingly, a single genome profile plate provides information equivalent to that of sequencing several kb (*10*). A genome profile consists of around 10 bands, each of which (~300 bp in average) can offer the information equivalent to that obtained by sequencing a DNA molecule of the same size with regard to determining if the DNA contains mutations.

The experimental errors in GPMA can be separated into two types: Errors A and B (Fig. 2B). The former consists of operational errors and a PCR-blurring error that is inevitably introduced by PCR since the *Taq* polymerase introduces random errors at a low rate. Therefore, a pair of genome profiles may be separated in ΔPaSS by this effect (<0.01 (*15*)) and the summating of Errors A and B is estimated to be ~0.01 in total while the latter can be taken as that of the spontaneous mutation. From the sequencing results, the maximum rate of spontaneous mutation was estimated as 2×10^{-4} per base per replication (Fig. 7). Considering that the replication error rates of *Taq* polymerase and *E. coli* are

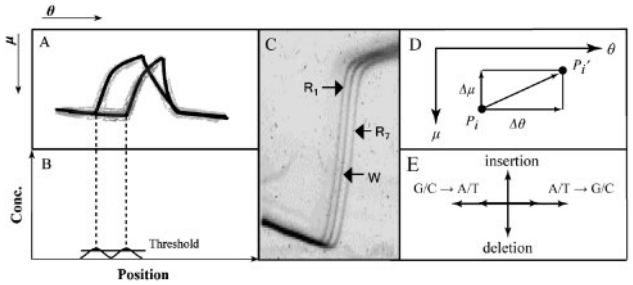

Fig. 8. **Schematic representation of *spiddos* shift.** The typical transition patterns in μTGGE of two DNA bands obtained before and after mutations are illustrated schematically (Panel A). Since each DNA band consists of a distribution of DNA sequences as drawn in Panel B (faint expanses in Panel A), feature points are restricted by the threshold level of the DNA concentration, *i.e.* the observable amount, indicating that a change of the DNA sequence distribution will result in a shift of the feature point. Panel C shows that a single point mutation leads to an observable band shift (from Band W to R_1/R_7; taken from (*38*) with permission). Note that the transition patterns in the denaturing gradient gel due to DNA melting are characteristic of their sequence (*39*). (D) The shift of *spiddos* can be decomposed to two elements of mobility (μ) and temperature (θ). (E) Horizontal shifts (direction of the axis θ) denote base substitutions, and vertical shifts (direction of the axis μ), insertion or deletion.

reportedly $\sim10^{-4}$ and $\sim10^{-6}$ per base per replication, respectively (*35–37*), this estimate for spontaneous mutation is reasonable but must be inflated by the errors caused by the *Taq* polymerase. GPMA appears to be capable of measuring the spontaneous mutation rate provided that a sufficient number of experiments have been carried out, and does so more easily than the sequencing method (Fig. 5). In any case, it is evident that although the sequencing approach can provide accurate data on a particular sequence, it requires further works to obtain statistically significant results by performing voluminous experiments.

Possible Detection Mechanism of GPMA—GPMA has proved to be functional and to have intrinsic merits as a mutation assay. Some aspects of the methodology require further consideration: (1) Why were such clear results obtained using a population, but not a clone, of bacteria? (2) Why was GPMA more sensitive and more informative than the sequencing approach employed here? Although both issues are very challenging and difficult to answer, we can speculate on possible answers: Queries 1 and 2 may be explained by the statistical behaviour of a population and the contribution of the SSCP (single-strand conformation polymorphism) phenomenon of ssDNAs during PCR, respectively (Supplementary Figs. S7 and S8). Since a large population of bacteria are involved, the DNAs obtained from them inevitably have differences in their sequences at the beginning of the experiments. The fate of the different sequences (*i.e.* bearing mutations of different types at different sites at different times) may vary during the course of experiment. As a result, we can compare two populations of DNA obtained from the pre- and post-culture of bacteria as shown in Fig. 8A, where, for the sake of simplicity, only single feature

points from two populations (which will be converted to *spiddos* by normalization) are superimposed. Both these points correspond to the tips of the broad distribution of DNAs shown in Fig. 8B which are made observable owing to the higher intensity than the threshold of visibility. Figure 8 contains an explanatory panel showing that a shift of a feature point (*spiddos*) can be caused by a point mutation (Panel C) (*38*). Thus, a population of DNAs can result in a broad distribution (*40*), suggesting the phenomenon observed in GPMA. As shown above, the feature point (leading to *spiddos*) can be defined to be the most intensive small area thus observed. Owing to this fact, the volume of sequence information contained in a population of DNAs can be reduced to the simple quantity of the coordinate of a *spiddos*. In consequence, the *spiddos* thus defined worked well for identification of mutations.

In contrast, no definite solution can be provided for Query 2. All we can speculate is the involvement of an SSCP phenomenon, similar to those recently shown to be a general phenomenon of single-stranded DNAs (*21*, *41*, *42*) (Supplementary Fig. S7). This phenomenon is explained by the structural dynamics of ssDNA in solution, which involves very unstable intramolecular interactions (*42*). We speculate that SSCP may expand the area for detection of point mutations beyond the DNA sequence area actually amplified, and point mutations result in changing the distribution of a population of DNA and shifting a *spiddos*. Since we do not know the rates of point mutations induced by mutagenic chemicals, we cannot measure how much contribution the SSCP phenomenon makes to the sensitivity of GPMA. In any case, the SSCP phenomenon must be involved in elevating the sensitivity of GPMA. The rationale of the mechanism related to SSCP phenomenon needs to be demonstrated somehow and does not mean that it can exclude any other possible mechanisms.

Finally, as stated above, the finding that even at concentrations of ppb, chemicals can be mutagenic is novel and significant. Recently, Matsui *et al*. (*43*) reported that AF2 was mutagenic at a ppb concentration. This fact urges reconsideration of the effect on our health and the ecosystem of long-term exposures to reagents at low concentrations, and thus, out of the range of detection by conventional mutation assays. Undoubtedly, the effect of a mutagen must vary between test organisms. Some chemicals only become mutagenic following microsomal processing. This must be a future challenge for the GPMA methodology since it does not depend on use of a specialized test cell. In other words, GPMA should be able to detect genetic alterations induced in any cell type. This opens the way to use of mouse and human cell lines for screening for effects on human health and for drug discovery. Thus, the novel approach presented here, GPMA, should be a useful mutation assay that both complements and reinforces the standard Ames test.

Supplementary data are available at *JB* online.

This study was supported partly by funding from the Rational Evolutionary Design of Advanced Biomolecules (REDS) Project, Saitama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence supported by JST.

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