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Elevated glucose 6-phosphate levels are associated with plasmid mutations *in vivo*

(reducing sugars/DNA adducts/aging/nonenzymatic glycosylation/reactive intermediates)

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ABSTRACT **The incubation *in vitro* of plasmid pBR322 DNA with glucose 6-phosphate (Glc-6-*P*) has been shown to have a mutagenic effect when the plasmid was transformed into wild-type *Escherichia coli*. To further investigate the modifications of DNA by the reducing sugar Glc-6-*P*, we have developed an *in vivo* model to monitor plasmid DNA mutations. *E. coli* strains that are defective for phosphoglucose isomerase (strain DF40) alone or phosphoglucose isomerase and glucose-6-phosphate dehydrogenase (strain DF2000) accumulate Glc-6-*P* when grown in gluconate minimal medium in the presence of glucose. These strains and the control strain K10 were transformed with pAM006, a plasmid that carries the genes for ampicillin resistance and β-galactosidase production, and grown for 24 hr under conditions that prompted the accumulation of Glc-6-*P*. An increase in plasmid mutations was observed (7- and 13-fold) that was associated with the increased intracellular levels of Glc-6-*P* (20- and 30-fold) present in the DF40 and DF2000 *E. coli* strains, respectively. Growth of the mutant bacteria in gluconate minimal medium does not increase the intracellular levels of Glc-6-*P* or the rate of plasmid mutations over background. Further characterization of the mutated plasmid DNA showed that insertions, deletions, and point mutations were responsible for the loss of β-galactosidase production. The increase in plasmid mutations as a function of increased intracellular Glc-6-*P* levels suggests that the accumulation of adducts formed by Glc-6-*P* and other reducing sugars may contribute to DNA damage.**

Reducing sugars can react with the amino groups of proteins over time to form stable covalent adducts that have characteristic physical properties of being fluorescent and yellow–brown in color (1). The formation of these pigments was first noted by Maillard (17) and has been extensively studied by food chemists in the intervening years. Within the past few years it has become apparent that this reaction is occurring in living tissues as well. The Amadori product has been found on many proteins, and the advanced glycosylation pigments have been found to accumulate over time on a number of long-lived proteins *in vivo* (2–5). The amino groups of DNA have also been shown to react nonenzymatically with reducing sugars to form yellow–brown pigments that have physical properties similar to those observed with proteins (6). Besides imparting a yellow–brown color to the DNA, these adducts, when formed *in vitro*, have been noted to decrease the transfection ability of f1 phage DNA (6) and prompt the formation of mutations on plasmid pBR322 DNA (7). The rate of chemical addition of the reducing sugars to double-stranded DNA was much slower than that observed between reducing sugars with single-stranded DNA or proteins (6). However, a reactive intermediate was found to be formed when reducing sugars were incubated with lysine or polyamines. These intermediates can react rapidly with either single- or double-stranded DNA to crosslink the amine to the DNA (8). These results led us to propose that the nonenzymatic reaction *in vivo* of reducing sugars with DNA directly or through protein crosslinks could adversely affect the functioning of DNA and possibly be responsible for DNA mutations noted during the process of aging.

Although calculations predicted that the rate and amount of the adducts formed would be sufficient to anticipate *in vivo* consequences, it was necessary to examine this hypothesis in an *in vivo* model. The current paper describes a model system that takes advantage of two *Escherichia coli* mutants (DF40 and DF2000) that lack either the enzyme phosphoglucose isomerase or both phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, respectively. These two mutants grow well with gluconate as their carbon source but can accumulate intracellularly, the reducing sugar glucose 6-phosphate (Glc-6-*P*) when glucose is present in the medium. Our objective was to measure the rates of mutation of a plasmid-carried marker gene (β-galactosidase) that was present for 24 hr in an *E. coli* host that had normal or elevated intracellular Glc-6-*P* levels. In comparison to the control strain K10, a 7- and 13-fold increase in plasmid mutation rate was observed in the DF40 and DF2000 strains when the accumulated Glc-6-*P* levels were increased 20- and 30-fold, respectively. No increase in mutation rate of the plasmid marker gene was observed if the mutant strains were grown under conditions that did not result in the accumulation of Glc-6-*P*. Analysis of the mutated plasmids revealed that most of the mutations resulted from insertions and deletions of the plasmid DNA.

MATERIALS AND METHODS

Bacterial Strains. The following K-12 *E. coli* strains were used: (*i*) K10 HFr [*tonA22, ompF626, relA1, pit-10, spoT1*, $T2^R$; (Coli Genetic Stock Center, New Haven, CT) CGSC 4234]. (*ii*) DF40 HFr [*tonA22, ompF626, relA1, pit-10, spoT1*, $T2^R$, *pgi-2*; CGSC 4861]. (*iii*) DF2000 HFr [*tonA22, relA1*, $T2^R$, *pgi-2, zwfA2*; CGSC 4863]. (*iv*) SB4288 F^- [(Δ *lac-proB, recA, thi-1, relA, mal-24, spc12, supE-50*, DE5]. Strains K10, DF40, and DF2000 were transformed with pAM006 by standard calcium chloride treatment (9).

Plasmids. The plasmids, pAM006 and pKM005, were a gift from P. Green (Rockefeller University, New York) and M. Inouye (Rutgers University, New Brunswick, N.J.). pKM005 carries the gene conferring ampicillin resistance (Amp^R) and inactive, promoterless *lacZ* and *lacY* genes (10). pAM006 was derived by the insertion of the *ompA* promoter upstream of *lacZ* to activate *lacZ* and *lacY* transcription (11).

Media and Buffers. M63 minimal medium was prepared as described by Miller (12) with the addition of 2% (wt/vol) glucose and gluconate in the mass ratios indicated or of 2%

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Abbreviations: Glc-6-*P*, glucose 6-phosphate; Amp^R, ampicillin resistance; Lac^-, lactose utilization deficient.

(wt/vol) gluconate, supplemented with ampicillin at 100 μg/ml. MacConkey agar (Difco) plates and LB broth (GIBCO) were prepared as directed by the manufacturers and supplemented with ampicillin at 100 μg/ml.

Growth Conditions. The transformed strains were grown in 500 ml of M63 medium containing gluconate alone or glucose and gluconate in a 9:1 mass ratio for 24 hr in a 37°C shaking water bath. Cultures were regularly checked for revertants in the mutant strains. Attempts to extend the experimental growth period beyond 24 hr led to an increased occurrence of revertants and prevented analysis. Under these conditions the growth rate of DF40 and DF2000 strains was slower by factors of 2 and 6, respectively, than the K10 control strain. The growth of the K10 strain did not change significantly when grown either in glucose/gluconate or gluconate minimal medium. Following growth, a sample was removed for Glc-6-*P* content analysis (see below); the remaining culture was harvested, and plasmid DNA was isolated as described by Maniatis *et al.* (9).

Detection of Plasmid Mutations. The purified plasmid from each strain was transformed separately into competent SB4288 cells that had been frozen as described by Hanahan (13) and plated out on MacConkey lactose indicator plates. Transformants from pAM006 and pKM005 were used as positive and negative controls, respectively. Those colonies exhibiting Amp^R but unable to ferment lactose were isolated, rescreened, and grown in LB broth overnight. The plasmid from each mutant colony was isolated on a small scale (9), linearized with *Xba* I, treated with RNase A (50 μg/ml), and electrophoresed on a 1.0% agarose gel containing ethidium bromide.

Glc-6-*P* Assay. Each culture was diluted to give an OD_{550} reading of 1.0 (10^8 cells per ml). Fifty milliliters of the diluted culture was centrifuged at 3000 × *g* for 30 min at 4°C. The pelleted culture was extracted with 5 ml of 6 M perchloric acid then 2 ml of 3 M perchloric acid. The pH of the combined supernatants was adjusted to 3.5 then assayed in triplicate. Glc-6-*P* was measured by the coupled reaction of glucose-6-phosphate dehydrogenase to NADPH production (14). Increase in absorbance was monitored at 340 nm.

RESULTS

All three strains (K10, DF40, and DF2000) are capable of utilizing gluconate as a sole carbon source (15, 16). When grown in gluconate minimal medium, neither of the mutant strains (DF40 and DF2000) accumulated significant levels of Glc-6-*P*; however, the presence of glucose in the medium led to the accumulation of Glc-6-*P*. Table 1 shows the concentrations of intracellular Glc-6-*P* found in each strain when grown for a 24-hr period in medium containing the glucose/gluconate mass ratios 9:1, 7:3, and 1:1. The 9:1 ratio of glucose to gluconate was chosen for the calculation of mutation rates since the concentration of intracellular Glc-6-*P* was highest at this ratio. Under these conditions the DF40 strain accumulated ≈20 times more Glc-6-*P* than the control strain K10, whereas DF2000 accumulated ≈30 times more. When the concentration of Glc-6-*P* was analyzed in the K10, DF40, and DF2000 strains after growth on gluconate minimal medium, no significant accumulation of Glc-6-*P* was detected.

Plasmid DNA was isolated and purified from each strain following growth on minimal medium containing gluconate alone or glucose/gluconate. The isolated plasmid DNA was used to transform the lactose utilization deficient (Lac^-) *E. coli* strain SB4288. The transformants were selected for Amp^R and screened for β-galactosidase production on MacConkey plates supplemented with ampicillin. Those colonies displaying an Amp^R/Lac^- phenotype were isolated and rescreened. The relative number of mutants per 10^5 transformants is given by the ratio of mutants found in the test strain (DF40 or DF2000) divided by the number of mutants found in the control strain (K10) (Table 2). The number of mutants increases proportionately in those strains that accumulated Glc-6-*P*. The relative number of mutants increased ≈7-fold with plasmid isolated from the DF40 strain and ≈13-fold with plasmid isolated from the DF2000 strain. This relationship is dependent on Glc-6-*P* since no increase is observed when the strains are grown in the absence of glucose.

To further characterize the mutations that had occurred as a result of elevated Glc-6-*P* levels, isolated plasmid DNA was analyzed from the Amp^R/Lac^- colonies. Table 3 summarizes the ratio of plasmid size changes per 10^5 transformants observed in the mutated plasmids from each strain. The background mutations in the K10 control strain and the plasmid mutations that originated from the DF40 strain show a predominance in plasmid size decrease. The mutations found in plasmid DNA isolated from the DF2000 strain appear to be distributed mainly between minor size changes and plasmid size increase. The cause or causes for variations in plasmid size ratios are not known at this time. Fig. 1 shows a representative sample of linearized, mutated plasmids isolated from each of the strains.

DISCUSSION

In the present communication, we have shown that raising the intracellular levels of Glc-6-*P* is associated with an increased rate of mutations of plasmid DNA. A comparison of Glc-6-*P* levels (Table 1) and plasmid mutation rates (Table 2) suggests a correlation between the intracellular Glc-6-*P* concentration and plasmid mutation rate. The control strain K10 shows minimal Glc-6-*P* accumulation over a 24-hr growth period in glucose/gluconate minimal medium, whereas the DF40 (phosphoglucose isomerase deficient) and DF2000 (phosphoglucose isomerase and glucose-6-phosphate dehydrogenase deficient) strains accumulate 20- and 30-fold

Table 1. Glc-6-*P* levels found in cells grown in minimal medium containing glucose/gluconate or gluconate alone

	Glc-6-*P*, μmol per 5 × 10^9 cells			
	Glucose/gluconate			
Strain	9:1 mass ratio*	7:3 mass ratio†	1:1 mass ratio†	Gluconate alone†
K10	0.028 ± 0.005	0.0177 ± 0.0031	0.0183 ± 0.0029	0.030 ± 0.003
DF40	0.553 ± 0.072	0.3478 ± 0.0230	0.1636 ± 0.0593	0.005 ± 0.002
DF2000	0.864 ± 0.011	0.6319 ± 0.0374	0.3230 ± 0.0796	0.004 ± 0.001

Overnight cultures of each strain grown in either gluconate or glucose/gluconate minimal medium were diluted to 10^8 cells per ml. Diluted culture (50 ml) was extracted with perchloric acid then assayed for Glc-6-*P* content.

*The results are means of triplicate experiments ± SD.

†The results are means of duplicate experiments ± SD.

Table 2. Relative mutation rates of cells grown in gluconate alone or glucose/gluconate

Strain	Relative *lac*⁻ mutagenesis per 10^5 transformants	
	Glucose/gluconate*	Gluconate alone†
K10	0.67 ± 0.47	0.5 ± 0.5
DF40	4.84 ± 0.65‡	1.0 ± 1.0
DF2000	8.71 ± 1.24‡	1.5 ± 0.5

Plasmid DNA (50 ng) isolated from cultures grown in gluconate or glucose/gluconate minimal medium was used to transform SB4288 competent cells. Colonies that were Amp^R but had a Lac^- phenotype were scored as mutants. Relative mutagenesis was determined by the ratio of mutants found in the mutant strains (DF40 or DF2000)/control (K10 strain).
*The results are means of triplicate experiments ± SD.
†The results are means of duplicate experiments ± SD.
‡The difference in mutation rate between K10 and the mutant strains DF40 and DF2000 is statistically significant ($P < 0.0001$).

more Glc-6-*P*, respectively. The relative mutation rates for each of the strains increase with intracellular Glc-6-*P* concentrations. A 13-fold increase in mutation rate occurs in the plasmid present in the DF2000 strain, whereas a 7-fold increase over background is observed in the DF40 strain. It seems likely that the increase in mutations is related to the higher intracellular Glc-6-*P* concentrations since growth of the mutant bacterial strains in gluconate minimal medium does not increase the intracellular Glc-6-*P* levels or the plasmid mutation frequency.

The *E. coli* strains K10, DF40, and DF2000 are not repair-deficient strains, suggesting that any mutations observed were those that were not repaired or were repaired incorrectly. Studies on the mutagenic effects of incubation *in vitro* of plasmid DNA with Glc-6-*P* indicate that some of the mutations that occurred were due to the attempts of the *E. coli* host to repair the plasmid DNA. When glycosylated plasmid DNA was transformed into a *uvrC*⁻ *E. coli* strain, no plasmid mutations were found. This is in strong contrast to the variety of insertions, deletions, and point mutations that were observed when the glycosylated plasmid was transformed into a wild-type strain (7). We have preliminary results that indicate that there is no significant induction of RecA when the bacteria are grown in gluconate minimal medium in the presence or absence of glucose (data not shown).

In the experiments described here, the number of mutations observed may be an underestimate of the actual number that have occurred. Some of the mutations that would have been overlooked in our assay include: mutations affecting the *E. coli* genome, mutations affecting Amp^R, plasmid replication, or more than one mutation in the *lacZ* and/or *lacY* genes, since a Lac^- phenotype on MacConkey plates does not distinguish between plasmids with defective lactose permease or β-galactosidase genes or both.

Table 3. Plasmid size changes

Size change	Fraction of total plasmids with size changes		
	K10	DF40	DF2000
>1-kb increase	3/12 (25.0%)	24/78 (30.8%)	75/153 (49.0%)
>1-kb decrease	8/12 (66.7%)	54/78 (69.2%)	27/153 (17.6%)
<1-kb change in either direction	1/12 (8.3%)	0/78 (0.0%)	51/153 (33.4%)

Plasmid DNA from phenotypic Amp^R/Lac^- colonies was isolated and linearized with *Xba* I. Samples were treated with RNase A and visualized on a 1.0% agarose gel containing ethidium bromide at 10 μg/ml. Twelve plasmids with size changes were isolated from 10^5 K10 transformants, 78 plasmids with size changes were isolated from 10^5 DF40 transformants, and 153 plasmids with size changes were isolated from 10^5 DF2000 transformants. Plasmids were grouped as indicated, and the percentage of the total plasmids isolated from each transformant strain is in parentheses.

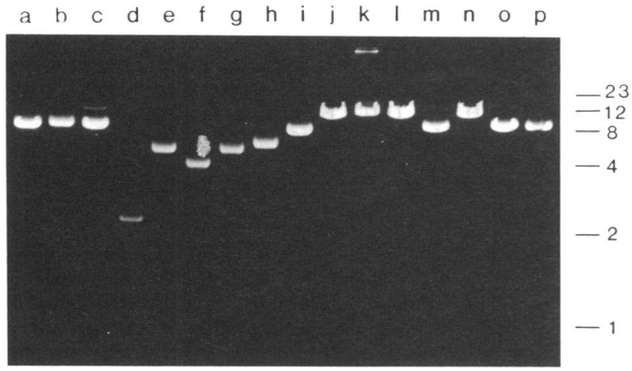

FIG. 1. Restriction analysis of mutated plasmid DNA from *E. coli* strains K10, DF40, and DF2000. Plasmid DNA was prepared on a small scale from 14 cultures of Amp^R/Lac^- colonies, digested with *Xba* I, and separated on a 1% agarose gel containing ethidium bromide at 10 μg/ml. Size standards are given as kilobase pairs. Lanes: a and p, pAM006; b–e, K10; f–i, DF40; j–o, DF2000.

The exact mechanism of the increased occurrence of the Lac^- mutants observed in bacterial strains that accumulate Glc-6-*P* cannot be defined with certainty at the present. Two obvious hypotheses are that the elevated intracellular Glc-6-*P* activates a recombination/repair mechanism that promotes mutations in the plasmid or that the Glc-6-*P* is reacting with the plasmid DNA to induce mutations by analogy to those observed following *in vitro* incubations of Glc-6-*P* and DNA. This latter mechanism could occur by direct addition of the Glc-6-*P* to the DNA or following the formation of a reactive intermediate with proteins or polyamines that can rapidly react with DNA (8). Although either of these adducts can form readily under *in vitro* conditions, they have not been identified under *in vivo* conditions.

The inevitability of the reaction over time of reducing sugars with proteins and DNA implies the necessity for biological systems to have evolved repair and replacement mechanisms for senescent molecules. Further studies of DNA repair following nonenzymatic glycosylation should give insight into the phenomenon of biological aging.

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