Hyperglycemia-Induced Embryonic Dysmorphogenesis Correlates With Genomic DNA Mutation Frequency In Vitro and In Vivo

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Congenital malformations affecting multiple organ systems are at least three times more common in infants of mothers with IDDM than in infants born to nondiabetic mothers. Numerous studies have confirmed the teratogenic effect of hyperglycemia on the developing embryo, although no direct mechanism has been determined. In this study, we aimed to correlate the frequency of *lac*I mutations with degree of hyperglycemic exposure and severity of malformations in mouse embryos from in vitro cultures. Day 8 transgenic mouse embryos cultured in 30 or 50 mmol/l glucose for 48 h exhibited a higher incidence of morphological abnormalities, as well as an increase in *lac*I mutation frequency, compared with embryos cultured in 10 mmol/l glucose with no abnormalities and a lower frequency of *lac*I mutations. We also used a transgenic *lac*I rat system to evaluate the relationship between abnormal embryonic development and DNA mutation frequency in day 11 embryos of severely diabetic rats (serum glucose >20 mmol/l). Compared with control embryos, the embryos from diabetic rats displayed significantly more malformations, shorter crown-rump lengths, fewer somites, and more than six times greater genomic DNA mutation frequency. Genetic analysis of the mutated *lac*I gene from both in vitro cultured mouse embryos and in vivo developed rat embryos revealed that the majority of mutations were due to base substitutions (transitions and transversions), but that the rate of large DNA mutations tended to increase in embryos exposed to a diabetic environment. Our results support the interrelationship between increased rates of congenital malformations and DNA mutations in the offspring of diabetic pregnancy. *Diabetes* 48:371–376, 1999

Chronic hyperglycemia has been implicated as one essential causative factor in the pathophysiological processes leading to a number of serious complications of the diabetic state, such as retinopathy, nephropathy, and neuropathy. Recent studies by the Diabetes Control and Complications Trial Research Group show that tight control of circulating glucose in IDDM decreases the progression of a number of diabetic complications. Unfortunately, even after near-normoglycemic circulating levels are achieved, preexisting organ and tissue damage is not readily reversed. Despite this recognition of the pathogenic potential of elevated glucose levels, the possibility of genetic contributions to these complications, in terms of accumulated somatic mutations, remains undetermined.

One particularly devastating pathogenic effect of hyperglycemia is diabetic embryopathy. Serious congenital malformations are at least three times more frequent in infants born to IDDM mothers, making birth defects the leading cause of perinatal deaths in this population (1,2). There is a direct correlation between the degree of maternal hyperglycemia during preconception and the first trimester, and the incidence and severity of fetal abnormalities (3–5). From human epidemiological studies, no specific diabetes-induced malformations have been identified, and all major organ systems appear to be susceptible, although increased frequency of some specific malformations has been noted in diabetic pregnancy. The best known example is the caudal regression syndrome, which is ~300 times overrepresented in offspring of diabetic mothers (2). Furthermore, the incidences of cardiac and neural malformations are increased in diabetic pregnancy (6–8).

Rodent embryo culture systems provide a method to analyze potentially teratogenic compounds under controlled conditions and have been used to identify specific teratogenic factors associated with the diabetic state (9–12). Embryos grown in culture media supplemented with serum from diabetic rats develop aberrations similar to those grown in vivo, and higher frequencies of dysmorphogenesis are directly related to the severity of diabetes of the serum donor (13–18). The potential teratogenic effect of elevated exogenous glucose alone has also been assessed in rodent embryo cultures. Rodent embryos cultured in media supplemented with glucose at levels comparable to those found in severe diabetes resulted in a glucose concentration–dependent increase in embryonic malformations, which emphasizes the critical role of elevated glucose in the development of physical abnormalities (9,11,19,20). Studies by us have implicated an important role for free radicals in diabetic embryopathies (11,21–24). The addition of free radical scavengers, i.e., superoxide dismutase or *N*-acetylcysteine, significantly reduces the teratogenic effects of hyperglycemia in rodent embryo cultures (11,18,21,22). More recently, the oral administration of antioxidants, i.e., butylated hydroxytoluene, vitamin E, or vitamin C, to diabetic rats was found to markedly reduce the frequency of embryonic malformations compared with diabetic rats that did not receive antioxidant therapy (25–29). These findings suggest that oxidative stress is involved in the etiology of embryonic dysmorphogenesis. The exact

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PCR, polymerase chain reaction; PFU, plaque-forming unit.

mechanism remains elusive; however, the findings may signify increased production of reactive oxygen species, diminished antioxidative defense capacity, or both.

The availability of certain transgenic rodent strains provides a unique opportunity to investigate the embryopathic effects of hyperglycemia at both the physical and molecular levels. Big Blue transgenic mice (C57BL/6) and rats (Fischer 344) that contain chromosomally integrated copies of the bacterial *lac*I gene in every cell have been developed and used to analyze the in vivo genotoxicity of numerous mutagenic agents (30–32). Because the *lac*I gene is neither positively nor negatively selected, it acts as a neutral mutagenesis reporter gene to detect DNA damage in a natural chromosomal environment in vivo. The *lac*I mutant frequency has been found to be comparable to that of the endogenous gene *dbl* (33). The in vitro and in vivo use of *lac*I$^+$ transgenic rodent embryos provides an effective model in which to evaluate the molecular mechanisms of teratogenic action of factors present in the diabetic maternal environment, specifically, hyperglycemia. This system is particularly sensitive to events with potential genotoxic or mutagenic consequences, since the embryo can be analyzed during critical periods of in utero development, allowing the molecular evaluation of events that would otherwise be difficult to identify. Our current investigation using model transgenic rodent embryos exposed to hyperglycemic conditions in vitro or in vivo focuses on the relationship between glucose-mediated developmental malformations and the presence of genomic DNA mutations in diabetic embryopathies.

RESEARCH DESIGN AND METHODS

In vitroembryo cultures. On day 8 of gestation, transgenic embryos from 13 B6D2/J F1 females mated with C57B/6 transgenic *lac*I$^+$ males (Big Blue mice; Stratagene, La Jolla, CA) were collected sterilely for in vitro culture. High spontaneous malformation rates have been observed when embryos are cultured at earlier stages, presumably reflecting the inherent fragility of younger embryos (21). Embryos were cultured as previously described (21). Briefly, isolated embryos with their surrounding yolk sac membrane were maintained at 38°C in polypropylene tubes containing 5 ml of culture medium consisting of 80% (vol/vol) rat serum and 20% (vol/vol) 0.9% (wt/vol) NaCl supplemented with glucose to achieve end concentrations of 10, 30, or 50 mmol/l. Culture tubes were gassed with different $N_2/O_2/CO_2$ gas mixtures at the start and after 24 and 40 h of culture, as previously described (21). The media were also changed after 24 h. Embryos were harvested, dissected out of their yolk sacs, and morphologically evaluated after 48 h of culture, corresponding to gestational day 10 in vivo. Embryonic development was assessed by measuring the crown-rump length (mm) and the number of somites. Embryos were morphologically scored based on visible malformations. A malformation score of 0 indicated a completely normal embryo, whereas a score of 1 showed only a minor abnormality, e.g., an open posterior end of the neural tube. A malformation score of 10 indicated an embryo with multiple malformations, such as an open neural tube, rotational defects, and/or an enlarged heart. After assessments, the embryos were flash-frozen for later DNA mutational analysis (see below).

In vivo rat embryo studies. At 1–2 weeks before mating, hyperglycemia was induced in female U strain rats by a single intravenous injection of streptozotocin (40 mg/kg), which was a gift from Pharmacia Upjohn (Kalamazoo, MI). The U strain is a Sprague-Dawley–derived rat strain. The outcome pregnancy is normal if the maternal metabolic rate is not impaired, but maternal diabetes yields offspring with congenital malformations (35). Animals with a serum glucose concentration >20 mmol/l 1 week after the injection (Glucose Analyzer 2; Beckman Instruments, Fullerton, CA) were considered diabetic. Rats were maintained on a 12-h light/dark schedule with free access to pelleted food (R36; Lactamin, Stockholm, Sweden) and tap water. Transgenic rat embryos were obtained after the overnight mating of 10 female U strain rats (diabetic and nondiabetic) with male Big Blue transgenic rats, where the presence of sperm in a morning vaginal smear designated gestational day 0. Pregnant rats were killed on pregnancy day 11, and embryos were dissected out of the uterine horns and evaluated for malformations, crown-rump length, and somite number before flash-freezing for later DNA mutational analysis (see below).

Genomic DNA isolation. Genomic DNA was prepared by Dounce homogenization of frozen embryos in buffer (136 mmol/l NaCl, 12 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, 2.6 mmol/l KCl, 10 mmol/l EDTA [pH 8.0]) containing RNase A (0.1 mg/ml) and digestion with proteinase K (2 mg/ml) in buffer containing 2% SDS and 50 mmol/l EDTA (pH 8.0) for 2 h at 50°C. Genomic DNA was additionally deproteinized with Tris-buffered phenol/chloroform (1:1, pH 8.0), chloroform/iso-amyl alcohol (24:1), and precipitated with ethanol. Precipitated DNA was redissolved in buffer containing 10 mmol/l Tris/1 mmol/l EDTA (pH 8.0) to give a final concentration between 0.5 and 1.0 mg/ml. DNA samples were then assessed for mutations to the *lac*I gene (30–32)

Mutagenesis assay. Shuttle vector DNA containing the *lac*I transgene was excised from 5 µg of purified genomic DNA by the addition of bacteriophage packaging extract (Transpack; Stratagene), resulting in the reconstitution of infective phage particles that contain a single copy of the *lac*I target gene. Bacteriophage particles were preabsorbed to *Escherichia coli* SCS-8 cells [*recA1, endA1, mcrA* Δ*(mcrBC-hsdRMS-mrr),* Δ*(argF-lac)U169,* ϕ*80dlacZ*Δ*M15, Tn10 (tet^r)*], mixed with top agarose containing the chromogenic substrate X-gal (5-bromo-4-chloro-3-indoyl β-D-galactopyranoside), and plated on NZY agar assay trays. Phenotypic *lac*I mutants were screened colorimetrically by visual detection of β-galactosidase production. Mutant plaques were enumerated and replated on fresh X-gal/NZY agar plates to confirm the *lac*I$^-$ phenotype. The mutant frequency was determined by the ratio of blue plaques to the total number of plaque-forming units (PFUs) screened for each fetus. Data are presented as the mutant frequency of each fetus (*lac*I$^-$ phenotype/100,000 PFU) determined by counting at least 100,000 PFU per sample. Typically, 10,000–20,000 PFU/µg genomic DNA are obtained.

Polymerase chain reaction analysis. Phagemids containing the *lac*I transgene were produced by in vivo excision of bacteriophage λ after addition of R408 helper phage. Phagemid DNA was recovered from the bacterial host by alkaline lysis and used as a template for DNA amplification as follows: 1 µl of DNA (1–2 µg) containing the *lac*I gene was added to 49 µl of a reaction mixture containing 45 µl of polymerase chain reaction (PCR) Supermix (Gibco, Grand Island, NY) and 2 µl each of the oligonucleotide primers. The 5′ sense primer (5′-GACACCATCGAATGGTG-3′) and the 3′ antisense primer (5′-ACATACGAGCCGGAAGC-3′) define a 1,250-bp fragment that spans the entire *lac*I coding sequence. After an initial 2 min denaturation step at 92°C, each cycle consisted of 1 min denaturation at 92°C, 1 min annealing at 47°C, and 1 min polymerization at 72°C. This program cycled 35 times and ended with a 10-min elongation step at 72°C. PCR products were analyzed for size differences by electrophoresis through 0.8% agarose gels containing 1 µg/ml ethidium bromide. By comparison with DNA molecular weight markers, DNA size changes of >20 bp were reliably detected.

DNA sequence analysis. DNA sequencing reactions were performed on mutant phagemid DNA using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied Biosystems 373A DNA sequencer. Briefly, 0.5 µg of phagemid DNA was added to a sequencing reaction containing 3.2 pmol of primer appropriate to the *lac*I gene and 8 µl of Terminator Ready Reaction Mix to give a final volume of 20 µl. Cycling reactions were performed in a Perkin-Elmer/Cetus Thermal Cycler Model 9600 (Norwalk, CT). After completion of 25 cycles, the sequencing reaction products were purified on Centri-Sep spin columns (Princeton Separations, Adelphia, NJ) to remove unincorporated DyeDeoxy terminators and dried in a vacuum centrifuge. Samples for sequence analysis were redissolved in 5 µl of a solution containing deionized formamide and 50 mmol/l EDTA (pH 8.0) in a ratio of 5:1, respectively. The samples were denatured at 90°C for 2 min before being loaded on a 6% acrylamide/50% urea sequencing gel that had been pre-run for 30 min. The sequencing samples were electrophoresed for 8–12 h. Data were collected automatically throughout the run and stored electronically. Sequence data were subsequently analyzed for correspondence to the wild-type *lac*I gene fragment.

Statistics. Differences between means were evaluated by one-way analysis of variance, where the applied test was Fisher's protected least significant differences (PLSD) test at the 95% confidence level, or with χ^2 statistics. Comparisons between different experimental groups were based on individual embryos, except for the evaluation of malformation score, for which the Fisher's exact χ^2 test for 2×2 tables was used.

RESULTS

Day 8 transgenic mouse embryos that had been cultured in vitro for 48 h in exogenous glucose (10, 30, or 50 mmol/l) were harvested and assessed for malformations, number of somites, and crown-rump length. Genomic DNA was isolated from each embryo and analyzed for *lac*I DNA mutations. As shown in Table 1, there is a statistically significant glucose

TABLE 1
Morphological and DNA mutation data of mouse embryos cultured in 10, 30, or 50 mmol/l glucose

Glucose (mmol/l)	Embryos (n)	Somites (n)	Crown-rump (mm)	Malformation score	Mutant frequency $\times 10^{-5}$	Minor DNA size change (%) (<20 bp)	Major DNA size change (%)	
							Loss (>20 bp)	Gain (>20 bp)
10	10	30.0 ± 0.7	4.0 ± 0.2	1.0 ± 1.0	2.8 ± 0.54	78	13	9
30	8	26.8 ± 2.0	3.2 ± 0.2*	3.9 ± 1.8	4.2 ± 0.87	70	19	11
50	12	22.8 ± 2.0†	2.9 ± 0.3†	7.6 ± 1.3†	5.5 ± 0.80*	57	39	4

Data are n, means ± SE, or %. *$P < 0.05$; †$P < 0.005$ for 10 vs. 30 or 50 mmol/l glucose.

concentration–dependent decrease in the number of somites and crown-rump length after culture. Thus, the somite number and crown-rump length are decreased in embryos that developed in 50 mmol/l glucose compared with those that developed in 10 mmol/l glucose, with the 30 mmol/l glucose-cultured embryos being numerically intermediate (Table 1). There was also a significant increase in the number of embryos displaying obvious malformations after culture in 50 mmol/l glucose compared with those cultured in 10 mmol/l glucose. A stepwise increase of embryonic dysmorphogenesis in proportion to increased ambient glucose concentration has been repeatedly observed in rodent embryos in vitro (9,18–20). The lack of a notable effect in somite number and malformation score in the 30 mmol/l glucose group in the present study therefore may be ascribed to small numbers of observations. In addition, however, there was an ~2-fold increase in *lac*I mutant frequency in genomic DNA isolated from embryos cultured in 50 mmol/l glucose ($5.5 \pm 0.80 \times 10^{-5}$) compared with DNA isolated from embryos that developed in 10 mmol/l glucose ($2.8 \pm 0.54 \times 10^{-5}$, $P < 0.05$), while a 1.5-fold increase was observed in embryos from the 30 mmol/l glucose ($4.2 \pm 0.87 \times 10^{-5}$) culture (Table 1).

Analysis of PCR products from mutant *lac*I clones obtained from embryos cultured in 10 or 30 mmol/l glucose demonstrated that >70% of the mutations were due to small (<20 bp) DNA alterations, while deletions and insertions >20 bp accounted for 20–30% of the mutations (Fig. 1). There was no difference between the proportions of small (<20 bp) and large (>20 bp) DNA mutations in the embryos cultured in 10 and 30 mmol/l glucose ($P > 0.1$). In contrast, more than 40% of the *lac*I mutants from embryos cultured in 50 mmol/l glucose tended to be due to large DNA mutations (10 vs. 50 mmol/l glucose-cultured embryos, $0.05 < P < 0.1$, χ^2 statistics), and therefore, the small base substitutions accounted for only 57% of the mutations in the 50 mmol/l glucose cultures (Table 1). Sequence analysis of randomly chosen clones from the 10 and 30 mmol/l glucose groups confirmed that frameshifts, transitions, and transversions accounted for the majority of the observed *lac*I mutant phenotypes (Fig. 1). Sequence analysis of random *lac*I mutant clones from embryos that developed in 50 mmol/l glucose showed that a proportion of the mutations were due to small deletions (5–15 bp), while the majority were due to frameshifts and base substitutions. There did not appear to be any specific susceptible regions of the *lac*I gene for mutagenesis nor did there appear to be any specific type(s) of sequence alterations (Fig. 1, Table 2).

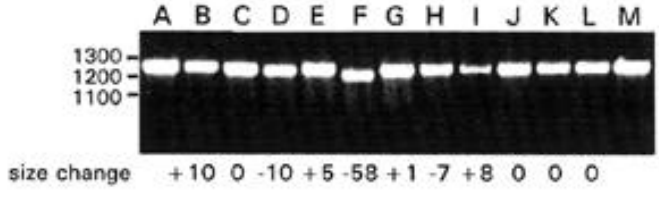

FIG. 1. PCR products from randomly selected *lac*I mutants from mouse embryos cultured in 10, 30, or 50 mmol/l glucose. *Lanes A* and *M*: *lac*I control; *lanes B–D*: 30 mmol/l glucose; *lanes E–I*: 50 mmol/l glucose; and *lanes J–L*: 10 mmol/l glucose. Size changes were determined by DNA sequence analysis.

To expand on our in vitro results with transgenic mice, we used a malformation-prone strain of rats to further investigate the interrelationship between hyperglycemia, malformation score, somite number, crown-rump length, and DNA mutations frequency in vivo. Day 11 transgenic rat embryos that developed in a normal or a diabetic maternal environment (glucose >20 mmol/l) were analyzed. Significant differences were observed between the embryos that developed under normal conditions and those that developed in a diabetic environment. The number of somites and crown-rump length were significantly lower in embryos harvested from diabetic rats than in those isolated from nondiabetic rats, and the incidence of malformations was dramatically increased (Table 3). There was a dramatic increase in *lac*I mutations in genomic DNA from embryos obtained from diabetic rats. A more than sixfold increase in phenotypic *lac*I mutations was observed in rat embryos that developed in a maternal diabetic environment ($14.2 \pm 2.2 \times 10^{-5}$) compared with embryos that

TABLE 2
DNA sequence analysis of *lac*I mutations

Mutant	Nucleotide no.	Mutation	Amino acid change
B	569	+10	Frameshift
C	343	G:C–T:A	Glu-stop
D	945–954	–10	Frameshift
E	634	+5	Frameshift
F	502–551	–50	Frameshift
G	905	+1	Frameshift
H	380–386	–7	Frameshift
I	376	+8	Frameshift
J	736	T:A–C:G	Leu-Pro
K	372	C:G–T:A	Leu-Phe
L	288	G:C–C:G	Arg-Pro

Data are from Fig. 1.

TABLE 3
Morphological and DNA mutation data of embryos from normal and diabetic rats

	Embryos (n)	Somites (n)	Crown-rump (mm)	Malformation score	Mutant frequency $\times 10^{-5}$	Minor DNA size (%) change (<20 bp)	Major DNA size change (%) Loss (>20 bp)	Major DNA size change (%) Gain (>20 bp)
Normal group	19	30.7 ± 0.6	4.5 ± 0.1	0.05 ± 0.05	2.2 ± 0.45	76	17	7
Diabetic group	17	24.6 ± 0.6*	3.2 ± 0.1*	3.5 ± 1.2†	14.2 ± 2.2*	59	36	5

Data are n, means ± SE, or %. *$P < 0.001$; †$P < 0.05$.

developed under normal conditions ($2.2 \pm 0.45 \times 10^{-5}$) (Table 3). PCR product analysis of the *lac*I mutant clones demonstrated that in both the diabetic and nondiabetic groups, the majority of mutants were due to base substitutions, but the rates differed markedly between the groups. Thus, the rate of small DNA damage (<20 bp) decreased numerically from 76% in control embryos to 59% in embryos of diabetic rats ($0.05 < P < 0.1$, χ^2 statistics), which corresponded to a doubling of the rate of large DNA deletions (from 17 to 36%) in the diabetic group (Table 3). Sequence analysis of random clones from both the diabetic and nondiabetic clones did not reveal any specific sequence change due to exposure to elevated glucose levels (Fig. 2, Table 4).

DISCUSSION

The most significant finding in the present study was the concomitant increase in the incidence of genomic DNA mutations and the rate of morphological dysmorphogenesis in mouse and rat embryos subjected to a diabetes-like environment in vitro and in vivo. This implicates that these processes are associated, but not species-dependent, and it suggests that the clarification of the relationship between them may yield new insights into the cellular processes controlling diabetic fetopathy.

Since the relationship between congenital malformations and maternal diabetes was first recognized, numerous human epidemiological and animal experimental studies have been reported (36). These studies have demonstrated an interrelationship between elevated maternal glucose levels during preconception and the first trimester, and subsequent birth defects. In humans, the incidence and severity of these malformations strongly correlates with mean HbA_{1c} levels during this time period (3–5). Despite the delineation of critical glucose levels and time periods at which the embryo is most affected, the precise mechanisms of hyperglycemia-induced pathogenesis remain largely unknown.

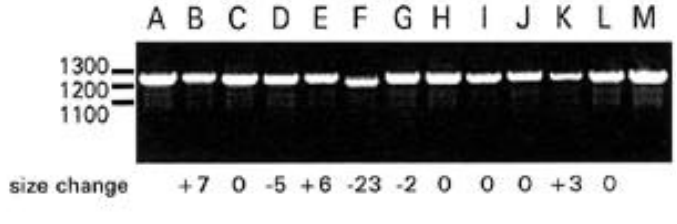

FIG. 2. PCR products from randomly selected *lac*I mutants from embryos of normal or diabetic dams. *Lanes A* and *M*: *lac*I control; *lanes B–H*: diabetic; *lanes I–L*: normal. Size changes were determined by DNA sequence analysis.

Data from our current investigations demonstrate that a strong positive correlation exists between hyperglycemia (in vitro or in vivo), somite number, crown-rump length, severity of malformations, and frequency and size distributions of *lac*I DNA mutations in our rodent embryo model systems. These results support previous observations that elevated glucose levels contribute to increased genomic DNA mutations (37) and congenital malformations in rodent embryos (9,11,19,20). Although the molecular mechanism(s) responsible for these consequences, whether interrelated or independent processes, has not yet been identified, there are several possible mechanisms.

Congenital abnormalities arising from DNA damage have been reported for a number of known mutagens and carcinogens (38,39). These molecules are relatively small and, like glucose, easily pass through the placental barrier to the fetus, where they can potentially react with fetal DNA. Some teratogens, such as iodide-containing drugs and methylmercury, are tissue-specific, targeting the fetal thyroid and central nervous system, respectively. There are, however, many chemicals, especially those interfering with normal metabolism, including DNA synthesis (e.g., 5-fluorouracil, mitomycin D), in which no specific embryopathic phenotype has been identified. The observed hyperglycemia-associated embryopathies could occur through indirect or direct glucose-mediated DNA damage through at least two possible mechanisms.

The diabetic state has been reported to induce elevated intracellular levels of oxygen free radicals, which may contribute to a number of diabetic complications including diabetic embryopathies. A number of studies have addressed this question and have demonstrated the protective effects of antioxidants on the developing embryo under hyperglycemic conditions in vitro and in vivo (11,18,21–29). The presence of antioxidants or oxygen free radical–scavenging enzymes in culture media protects rodent embryos from growth retardation and embryonic malformations ordinarily observed because of development in hyperglycemic medium (11,18,21,40). The types of *lac*I mutations observed in the present investigation could have arisen from incorrectly repaired free radical damage as the result of decreased DNA repair efficiency. However, further studies are needed to determine where the beneficial effects of antioxidants occur at the molecular level.

Glucose itself has been implicated in a number of diabetic complications, primarily through products formed during the Maillard reaction or nonenzymatic glycation of proteins and other macromolecules containing free amino groups (41,42). Glucose-derived modifications or advanced glycation end products have been shown to possess the ability to cross-link macromolecules inter- and intramolecularly. Glucose-modi-

TABLE 4
DNA sequence analysis of *lac*I mutations

Mutant	Nucleotide number	Mutation	Amino acid change
B	442	+7	Frameshift
C	941	T:A–A:T	Leu-stop
D	1073–1077	–5	Frameshift
E	341	+6	Insertion of two amino acids (Ala,Thr)
F	259–281	–23	Frameshift
G	852–853	–2	Frameshift
H	58	G:C–A:T	Ala-Thr
I	97	G:C–C:G	Val-Leu
J	773	C:G–T:A	Gln-stop
K	105	+3	Insertion of one amino acid (Val)
L	275	C:G–T:A	Ala-Val

Data are from Fig. 2.

fied proteins have been associated with atherosclerosis, nephropathy, and neuropathy, and their presence in fetal tissue has also been correlated with higher frequencies of congenital malformations (43). The modification of DNA by glucose in vitro has resulted in an unexpected mutational spectrum, ranging from simple base substitutions to large deletions to insertions due to duplications and transpositions. Data from a previous study suggested that DNA modification by the complex formation of glucose-derived adducts could be involved in the generation of congenital malformations (37). This conclusion was reached based on the presence of large deletions and insertions in sequenced *lac*I mutants. However, in contrast to the current study, these results were obtained from full-term fetuses that had been exposed to moderate hyperglycemia during the entire gestational period. More recently, elevated levels of 3-deoxyglucosone, a known intermediate of the Maillard reaction, have been found in rodent embryos cultured in elevated glucose concentrations for 48 h (44). Given the high reactivity of 3-deoxyglucosone with macromolecules (45,46), its formation and accumulation in embryos after culture in elevated exogenous glucose suggest that it may play a significant role in diabetic embryopathies. In support of this notion, 3-deoxyglucosone proved to be teratogenic to cultured embryos when added in quantities corresponding to those seen after exposure to high glucose (44). Furthermore, addition of superoxide dismutase to the 3-deoxyglucosone–exposed cultures diminished the rate of embryonic maldevelopment (44), thus indicating an interrelationship between the oxidative and glycation pathways for disturbed embryogenesis.

In this context, it should be pointed out that hyperglycemia in vitro and diabetes in vivo induce changes in embryonic metabolism other than increased DNA mutations, increased concentration of 3-deoxyglucosone, and the postulated state of reactive oxygen species excess. Thus, pronounced disturbances in arachidonic acid/prostaglandin metabolism have been documented (47–49), ultimately resulting in lowered prostaglandin E_2 levels (50), as well as changes in inositol and sorbitol metabolism (48,51). All of these alterations may have associations with the DNA mutations described herein, thereby illustrating the multifactorial nature of diabetic embryopathy.

In the current study, we have shown a correlative relationship between an increased incidence of DNA mutations, malformations, and growth retardation in rodent embryos and exposure to a hyperglycemic environment. The exact nature of this relationship, however, remains to be defined. From the available data, it appears that there is more than one mechanism involved in the increased DNA mutation frequency in the embryos associated with maternal hyperglycemia. A combination of glycation and oxidation, also referred to as glycoxidation (52), may be responsible for this increase. In addition, inefficient DNA repair may also contribute to the observed increase in genomic DNA mutations. Further investigations are warranted to fully elucidate the relationship(s) between elevated glucose levels and embryonic growth retardation, malformations, and genomic DNA mutations.

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