High-level and Erythroid-specific Expression of Human Glucose-6-phosphate Dehydrogenase in Transgenic Mice*

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Human Glc-6-P dehydrogenase (Glc-6-P) cDNA spanning the entire coding region was subcloned into a pSG5 vector that contains an early SV40 promoter, intron II of the rabbit β-globin gene, and a polyadenylation signal. This expression cassette was then placed downstream of the human β-globin locus control region and injected into fertilized mouse eggs. Among five transgenic founders that contained intact copies of the construct, one founder expressed human Glc-6-P dehydrogenase enzyme in a high-level and erythroid-specific fashion (5 x higher than endogenous Glc-6-P dehydrogenase activity). When this male founder mated with a normal individual, all the offspring that carried the transgene showed high-level expression of Glc-6-P dehydrogenase activity in erythroid cells. The endogenous mouse Glc-6-P dehydrogenase in all high-expression mice could be competed out by forming a hybrid with human Glc-6-P dehydrogenase. Our results indicate that the locus control region can drive the human Glc-6-P dehydrogenase gene to be specifically expressed in the erythroid cells of transgenic mice. The results described here provide a basis for experiments designed to express human Glc-6-P dehydrogenase in transgenic mice and suggest a suitable approach to producing a mouse model for studying human Glc-6-P dehydrogenase deficiency.

Glucose 6-phosphate dehydrogenase (Glc-6-P) is the first and key enzyme of the pentose phosphate pathway. Its physiological role is to provide NADPH, a source of reducing power that maintains sulfhydryl groups and aids in the detoxification of free radicals and peroxides and in a number of biosynthetic reactions. Glc-6-P dehydrogenase deficiency is the most common human enzymopathy, affecting 200 million people worldwide. Most Glc-6-P dehydrogenase-deficient patients are asymptomatic. Some are associated with acute hemolysis. Some are associated with acute hemolysis. Some are associated with acute hemolysis. The primary effect of Glc-6-P dehydrogenase deficiency occurs in red blood cells since the pentose phosphate pathway is the only source of NADPH and red cells contain no mitochondria. Third, the endogenous Glc-6-P dehydrogenase activity should be competed out to reduce the wild type effect. Finally, the inserted gene should be transmitted into the offspring of transgenic animals and should be expressed in their erythroid cells. To achieve these goals, we have designed and constructed an expression vector. This vector contains a SV40 early promoter and a polyadenylation signal to increase in vivo expression of cloned inserts. Intron II of the β-globin gene which may facilitate splicing of the expressed transcript (4), and a 2.5-kb cassette containing the human β-globin locus control region (LCR) (5) that drives downstream-inserted DNA to be specifically expressed in erythroid cells were also included. Using this vector we have successfully generated a transgenic line that carries a normal human Glc-6-P dehydrogenase gene. The production and expression of human Glc-6-P dehydrogenase in this line fulfill the criteria stated above. The results presented here may thus provide an experimental basis for the generation of an animal model that carries a mutated Glc-6-P dehydrogenase gene for studying Glc-6-P dehydrogenase deficiency.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The cDNA that spans the entire coding region of human Glc-6-P dehydrogenase was constructed by polymerase chain reaction amplifying the 530-base pair fragment that covers the N-terminal end of Glc-6-P dehydrogenase from a Molt-4 cDNA library. The amplified DNA was ligated into a pGPD-2A vector (obtained from ATCC; deposited by M. Persico) that contained the C-terminal part of human Glc-6-P dehydrogenase. The EcoRI fragment containing the entire coding sequence for human Glc-6-P dehydrogenase was subcloned into a pSG5 vector (Stratagene), an eukaryotic expression vector that contains an early SV40 promoter, intron II of the rabbit β-globin gene, and a polyadenylation signal. The entire coding sequence of human Glc-6-P dehydrogenase cDNA...
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was confirmed by DNA sequence analysis. The Glc-6-P dehydrogenase expression cassette in pSG5 was released by SalI digestion and then cloned into a µLCR plasmid (5) (a gift from G. Stamatoyannopoulos, Seattle) that contains four hypersensitive sites I-IV of the human β-globin LCR (the final product from the above construction was named the G6PDSLFS plasmid). The Not1/XhoI DNA fragment (5.5 kb) excised from the G6PDSLFS plasmid was used for microinjection experiments.

Generation of Transgenic Mice and Glc-6-P Dehydrogenase Activity Assay—Transgenic mice containing the human Glc-6-P dehydrogenase transgene were produced by microinjection of DNA into the male pronuclei of fertilized mouse eggs (CBA X C57BL), which were then transferred to pseudopregnant females (ICR) as described (6). The Glc-6-P dehydrogenase activity in the erythroid cells of transgenic mice (Tg) was measured as described (7) or by Glc-6-P dehydrogenase diagnostic kit (Sigma).

DNA Analysis—Mouse genomic DNA was extracted from tail biopsies as described (6). 10 µg of DNAs from normal mice and from Tg founders and their offspring were digested with BamHI, separated by agarose gel electrophoresis, and transferred to a Nitran filter (Schleicher & Schuell). The filter was then hybridized with the Not1/XhoI fragment labeled by a multiprime DNA labeling kit (Amer sham). The conditions for Southern analysis were as previously described (8) with modifications. In brief, the prehybridization and hybridization were performed in 0.5 M sodium phosphate, 7% SDS, 1% non-fat milk, 2.5 mM EDTA, and 100 µg/ml denatured salmon sperm DNA at 65 °C for 4 h and overnight, respectively. The filters were then washed once in 2× SSC, 0.1% SDS at room temperature and three times in 0.1× SSC, 0.1% SDS at 68 °C. Copy numbers of human Glc-6-P dehydrogenase were determined by dot blotting and laser densitometry as described (6).

Cellogel Analysis—Hemolysates were prepared as described (9). 15 µl of blood hemolysate were loaded on Cellogel (Serva, Germany). Electrophoresis was carried out in 0.1 M Tris borate, pH 8.1, 10 mM NADP at 200 volts for 50 min at 4 °C. Cellogel strips were stained in a 1-ml solution containing 0.1 M Tris-HCl, pH 7.0, 1 mM Glc-6-P, 1 mM MgCL2, 200 µM NADP, 40 µg of phenazine methosulfate, and 200 µg of nitro blue tetrazolium (10). The staining reaction was stopped by washing the strips in running water and then stored in 5% acetic acid solution.

RESULTS AND DISCUSSION

Fig. 1 illustrates the Not1/XhoI DNA fragment of a plasmid G6PDSLFS (see "Experimental Procedures" for details) that was injected into fertilized mouse eggs. A total of 133 fertilized eggs were injected and 18 mice were born alive (Table 1). Tail DNAs were extracted from these mice and assayed for the integration of the human Glc-6-P dehydrogenase gene by dot blot hybridization and Southern blot analysis. High-level expression of the functional human Glc-6-P dehydrogenase gene in the erythroid cells of transgenic mice (Tg) was detected by analyzing the Glc-6-P dehydrogenase enzyme activity and by Cellogel electrophoresis techniques. Mice which were positive for both DNA integration and high-level expression of Glc-6-P dehydrogenase gene served as founder animals for establishment of a transgenic line.

Of 18 mice examined, 5 were positive for integration of the transgene. The copy number of the transgene in positive mice, which varied from two to eight (Table 1), was determined by quantitative analysis of dot blot hybridization intensity on a Nitran membrane. Southern blot analysis of the genomic DNA from these transgenic mice revealed that the Not1/XhoI Glc-6-P dehydrogenase fragment was most likely to be integrated in a tandem head-to-tail fashion at a single (3013, 3016) or two (3002, 3007, 3015) chromosomal loci in founder animals. Fig. 2 shows that digestion of the genomic DNA with BamHI, which cuts three times within the injected Not1/XhoI fragment (Fig. 1), gave two predicted fragments (3.7 and 1.5 kb) and one or two fragments with variable size when the same Not1/XhoI DNA fragment was used as a probe for Southern analysis. The variable size of these fragments was caused by another BamHI site located near the integration site of cellular DNA. For example, a DNA fragment (2.9 kb) with a weak hybridization signal was detected in founder 3016 (Fig. 2A, lane 7), which may indicate the presence of a single chromosomal integration locus. The weak signal of this fragment was due to the shortness of the homologous region (only 0.15 kb long) between the BamHI-digested fragment (2.9 kb) and the DNA probe. A similar Southern pattern was observed in founder 3013. In this case, a 5.0-kb BamHI-digested fragment, instead of a 2.9-kb fragment, was observed (lane 5). This result suggests that the integration sites in these two founders are different. In addition, two fragments (5.8 and 3.6 kb) with weak hybridization signals were detected in normal individuals (lanes 2 and 8). These fragments appear to be a mouse Glc-6-P dehydrogenase gene picked up by crosshybridization with the human cDNA probe. Besides the 1.5- and 3.7-kb DNA fragments found in all founders, two other BamHI-digested fragments with variable size were also detected in three other founders (3002/lane 3, 3007/lane 4, and 3015/lane 6). These two fragments were probably generated by integration of the transgene into two different chromosomal loci. This observation was further confirmed by Southern analysis of the genomic DNAs obtained from founder 3007 and its offspring. The 3.0- and 2.4-kb BamHI-digested fragments identified in Tg3007 were transmitted either separately (0103/lane 10, 0107/lane 12, 0110/lane 13) or together (0104/lane 11 and 0115/lane 14) into its offspring in a Mendelian fashion (Fig. 2B). Glc-6-P dehydrogenase is an X-linked enzyme. However, our results (Fig. 3) show that the transgene can be transmitted from male (3007) to either female (0103, 0110, 0115) or male (0104), which suggests that the transgene was integrated into mouse autosomes. For convenience, the 2.4-kb BamHI fragment is named as A1 allele and the 3.0-kb fragment as A2 allele. Southern blot analysis revealed that no gross DNA rearrangement, insertion, or deletion occurred within the transgenes of the founders and their offspring.

In order to detect high-level expression of human Glc-6-P dehydrogenase activity, we analyzed the Glc-6-P dehydrogenase activity in the erythroid cells of transgenic mice (Table 1). The normal range of endogenous Glc-6-P dehydrogenase activity for normal F1 (CBA/C57BL6) mice (aged >6 weeks) at 30 °C in our laboratory was established to be from 14 to 24 IU/g Hb. Table I shows that one (3007) out of five founders revealed high levels of Glc-6-P dehydrogenase activity (>100 IU/g Hb). This male founder was therefore chosen to establish a transgenic line by mating with normal individuals.

Of 15 F1 progeny analyzed, five which were positive for integration showed high-level Glc-6-P dehydrogenase activity (Fig. 3). Interestingly, the Glc-6-P dehydrogenase activity in these mice appears to be expressed in both a tissue-specific and a copy number-dependent manner. Fig. 3 shows that the offspring (0104 and 0115) that carried both A1 and A2 alleles

![Fig. 1. Map of the G6PDSLFS construct used in transgenic experiments](top). The restriction map and the predicted size of *BamHI* fragments used for Southern analysis are also shown at the bottom. B, *BamHI*; S, *SalI*; E, *EcoRI*. G6PD, Glc-6-P dehydrogenase.
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TABLE I
A summary of the generation of transgenic founders

The normal mouse Glc-6-P dehydrogenase activity (aged > 6 weeks) at 30 °C was between 14 and 24 IU/μg Hb. The Glc-6-P dehydrogenase activity of founder 3007 was roughly estimated because this founder died 8 weeks after birth for unknown reasons.

| Plasmids injected | No. embryos transferred | No. mice born | No. Tg mice | Tg | Sex | Copy no. | Glc-6-P dehydrogenase activity IU/μg Hb |
|-------------------|-------------------------|---------------|-------------|----|-----|---------|-----------------------------------------|
| G6PDLSF           | 133                     | 18            | 5           | 3002 | M   | 8       | 14.3                                    |
|                   |                         |               |             | 3007 | M   | 4       | >100.0                                  |
|                   |                         |               |             | 3013 | F   | 2       | 22.6                                    |
|                   |                         |               |             | 3015 | M   | 5       | 14.4                                    |
|                   |                         |               |             | 3016 | F   | 2       | 18.5                                    |

FIG. 2. Southern blot analysis of human Glc-6-P dehydrogenase in transgenic founders (A) and their offspring (B) using the NotI/XhoI DNA fragment as a probe. A, M: BamHI-digested G6PDLSF plasmid DNA. Two predicted sizes of fragments (3.7 and 1.5 kb) with high intensity and a 3.1-kb fragment with low hybridization signal were detected by Southern analysis. The 3.1-kb fragment contains a 0.15-kb sequence (BamHI/XhoI) identical with the probe plus another 2.95-kb fragment derived from a pBluescript vector. N, BamHI-digested normal mouse DNA. The films of A and B were exposed for 6 and 12 days, respectively, in order to bring out the bands with low intensity.

FIG. 3. The pedigree of transgenic 3007 founder revealing the transmission and expression of human Glc-6-P dehydrogenase enzyme activity. Half-solid and open symbols represent heterozygous and normal individuals, respectively. Squares represent males and circles represent females. Slashed half-solid square indicates founder Tg3007 which died 8 weeks after birth. A1 and A2 alleles were determined by Southern blot analysis (see text for detail). The Glc-6-P dehydrogenase activity was determined as described under "Experimental Procedures" and shown in parentheses. Tg0103 and 0104 were killed 8 weeks after birth for analysis of Glc-6-P dehydrogenase activity in various tissues.

FIG. 4. Cellulose acetate electrophoresis of the erythroid Glc-6-P dehydrogenase of human and transgenic mice. M, normal mouse Glc-6-P dehydrogenase. H, human Glc-6-P dehydrogenase. H/M, human/mouse Glc-6-P dehydrogenase hybrid. A triple-banded pattern was observed in high-level expression Tgs (0103, 0104, and 0107), but not in a nonexpression Tg (3002).
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dehydrogenase activity to that of 0107 mother. Interestingly, the high-level expression of human Glc-6-P dehydrogenase activity seemed to be restricted to red cells only, since this increase effect was not detected in other mouse tissues analyzed (including heart, liver, kidney, and spleen, data not shown). The hematological data (including white blood cell count, red blood cell count, Hb concentration, hematocrit, mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin concentration) of all high-level expression mice revealed no significant difference from normal mice (data not shown).

Electrophoretic patterns of human and murine endogenous Glc-6-P dehydrogenase enzymes in the erythrocytes of transgenic mice were analyzed by cellulose acetate gel electrophoresis. Human Glc-6-P dehydrogenase B (Fig. 4, lane 6) and mouse Glc-6-P dehydrogenase (lane 2) are well resolved by electrophoresis, since the latter has the greater mobility. Fig. 4 shows that a triple-banded pattern with a different degree of intensity was observed in the erythrocytes of high-expression Tgs (0013/lane 3, 0104/lane 4, and 0107/lane 5), but not in Tg0902 (lane 1), a nonexpression founder containing 8 intact copies of human Glc-6-P dehydrogenase gene within its genome (Table 1). The major band with the highest intensity represents human Glc-6-P dehydrogenase enzyme, whereas the intermediate band is presumably a human/mouse heterodimeric enzyme. The third band with the fast mobility and the least intensity represents the mouse endogenous Glc-6-P dehydrogenase enzyme. Interestingly, most (Tg0103/lane 3, Tg0107/lane 5) or nearly all (Tg0104/lane 4) mouse Glc-6-P dehydrogenase was converted to the human/mouse hybrid and the expression level of endogenous mouse Glc-6-P dehydrogenase seemed to depend on the quantity of human Glc-6-P dehydrogenase expressed in Tgs. For example, Tg0104 (116 IU/g Hb) which revealed 5-fold higher Glc-6-P dehydrogenase activity than normal mice (14–24 IU/g Hb) had no detectable endogenous Glc-6-P dehydrogenase in its erythrocytes (Fig. 4, lane 4), whereas Tg0103 (56.5 IU/g Hb) and Tg0107 (54.0 IU/g Hb) which showed 2–3 times higher Glc-6-P dehydrogenase activity than normal mice still had a trace of endogenous Glc-6-P dehydrogenase expressed in their red cells. This result implies that the endogenous mouse Glc-6-P dehydrogenase molecules in transgenic erythrocytes can be completely competed out to form hybrid molecules with their human counterparts, once the expression level of the human Glc-6-P dehydrogenase reaches a threshold level (e.g. >5x higher than endogenous Glc-6-P dehydrogenase activity).

Similar results were observed in other high-level expression Tgs (data not shown). Heterodimeric Glc-6-P dehydrogenase has been observed in human-hamster (11), human-monkey (12), and human-mouse (13) somatic nonerythroid cell hybrids. The relative proportions of various dimeric enzymes could be generated by the relative ratios of the human and murine subunits synthesized and/or by the relative affinities between human and murine homo- or heterosubunits.

It has been reported that the LCR confers the attached genes in a high-level position-independent and erythroid-specific expression pattern in both transfected cell lines (4, 14–16) and transgenic mice (16–19). However, in our study, only one of five founders exhibited high-level and erythroid-specific expression. The other four, although they did contain intact copies of the construct as confirmed by Southern analysis, did not express human Glc-6-P dehydrogenase enzyme. The reasons for this effect are not clear at this moment. The position effect (the position of integration sites) or some other unknown mechanism may contribute to this phenomenon.

We (7, 20) and others (21, 22) have recently identified that at least seven types of mutations are responsible for Glc-6-P dehydrogenase deficiency in Chinese, but little is known about the relationship between clinical phenotype and molecular defects in this disease. In this study, we have generated a transgenic line that allows for high-level expression of a functional human Glc-6-P dehydrogenase gene in transgenic mouse erythrocytes. The highly expressed human Glc-6-P dehydrogenase in erythrocytes cannot only be transmitted from the founder to its offspring, but may also be used for the replacement of endogenous murine Glc-6-P dehydrogenase, which may reduce the “wild type” effect. The LCR/Glc-6-P dehydrogenase system described here will enable us to introduce any mutated Glc-6-P dehydrogenase genes into intact animals and generate transgenic lines to further study the pathophysiology of Glc-6-P dehydrogenase deficiency in the near future.

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