The EMBO Journal vol.14 no.21 pp.5209–5215, 1995

Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress

Pier Paolo Pandolfi[1,2], Fatima Sonati[1,3], Roberta Rivi[1,2,4], Philip Mason[1], Frank Grosveld[5] and Lucio Luzzatto[1,2]

[1]Department of Haematology, Royal Postgraduate Medical School, Ducane Road, London W12 0NN, UK, [2]Department of Human Genetics, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA and [5]Department of Cell Biology and Genetics, Faculty of Medicine, Erasmus University, 3000 DR Rotterdam, The Netherlands
[3]Present address: Department of Clinical Pathology, UNICAMP, Campinas, San Paulo, Brazil
[4]On leave of absence from: Department of Experimental Medicine, University La Sapienza, Rome, Italy

Glucose 6-phosphate dehydrogenase (G6PD) is a housekeeping enzyme encoded in mammals by an X-linked gene. It has important functions in intermediary metabolism because it catalyzes the first step in the pentose phosphate pathway and provides reductive potential in the form of NADPH. In human populations, many mutant G6PD alleles (some present at polymorphic frequencies) cause a partial loss of G6PD activity and a variety of hemolytic anemias, which vary from mild to severe. All these mutants have some residual enzyme activity, and no large deletions in the G6PD gene have ever been found. To test which, if any, function of G6PD is essential, we have disrupted the G6PD gene in male mouse embryonic stem cells by targeted homologous recombination. We have isolated numerous clones, shown to be recombinant by Southern blot analysis, in which G6PD activity is undetectable. We have extensively characterized individual clones and found that they are extremely sensitive to H_2O_2 and to the sulfydryl group oxidizing agent, diamide. Their markedly impaired cloning efficiency is restored by reducing the oxygen tension. We conclude that G6PD activity is dispensable for pentose synthesis, but is essential to protect cells against even mild oxidative stress.

Keywords: embryonic stem cells/glucose 6-phosphate dehydrogenase/housekeeping gene/knock out/oxidative stress

Introduction

The function of intracellular enzymes has traditionally been assessed by two independent and complementary methodologies: the biochemical approach and the genetic approach. On the one hand, the characterization of substrates and products identifies the biochemical role of an enzyme, particularly if it catalyzes one of the reactions in a defined metabolic pathway. On the other hand, the phenotypic characterization of natural mutants (Luzzatto and Mehta, 1995) or of artificially produced 'knock-out' mutants helps to pinpoint which functions are compromised when an individual enzyme is lacking. Whereas some enzymes are tissue-specific, many are ubiquitously expressed, and thus the genes encoding them qualify as 'housekeeping' genes (Singer and Berg, 1991; Larsen *et al.*, 1992). In the case of a tissue-specific gene, the consequences of gene ablation must usually be assessed in the appropriate tissue of a mouse reconstituted from the targeted cells. Although this approach has not yet been used for housekeeping enzymes of intermediary metabolism, it is in fact very attractive because the consequences of the complete lack of an enzyme can be seen in any cell.

Glucose 6-phosphate dehydrogenase (G6PD) is a housekeeping enzyme, highly conserved in evolution (Vulliamy *et al.*, 1992; Kletzien *et al.*, 1994), the biochemistry of which is well known (Luzzatto and Testa, 1978; Levy, 1979). It is conventionally referred to as the first and rate-limiting enzyme of the pentose phosphate pathway in all cells (Stryer, 1988), and it is therefore regarded as important in the biosynthesis of the sugar moiety of nucleic acids. At the same time, in mammalian cells G6PD provides reductive potential in the form of NADPH (Stryer, 1988). G6PD deficiency, resulting from mutations in the G6PD gene, is well known in humans as a common cause of pathology, specifically of hemolytic anemia (Beutler, 1978; Dacie, 1985; Luzzatto, 1993). In many of these mutants, G6PD deficiency is not extreme and the clinical manifestations are limited, as they occur only when an exogenous agent triggers hemolysis. In a few cases the hemolytic anemia is instead chronic and severe, and sometimes G6PD deficiency affects not only red blood cells but also white blood cells, causing a decreased capacity to kill bacteria (Gray *et al.*, 1973). Even in these cases, G6PD deficiency is never complete. The reason for this has been clarified by the analysis of naturally occurring G6PD mutations, of which ~70 are known (Vulliamy *et al.*, 1993). Whereas mutations in tissue-specific genes (such as those responsible for thalassemia, cystic fibrosis or muscular dystrophy; Kazazian, 1990; Tsui, 1992; Monaco, 1993; Tinsley *et al.*, 1993) include numerous large deletions, frameshifts and splicing defects, all G6PD mutations are missense or small in-frame deletions (Vulliamy *et al.*, 1993). Therefore it has been surmized that null mutations in the G6PD gene might be lethal, but this has not been formally proved. Definitive evidence as to the essential role of the enzyme is not available. Gene ablation is clearly the method of choice to determine whether the complete inactivation of G6PD is compatible with cell survival and growth, and to test whether it is essential for pentose synthesis, for defense against oxidative stress, or both. Here we report the results of such a test. We found that G6PD null cells are viable, but they are exquisitely sensitive to oxidative stress.

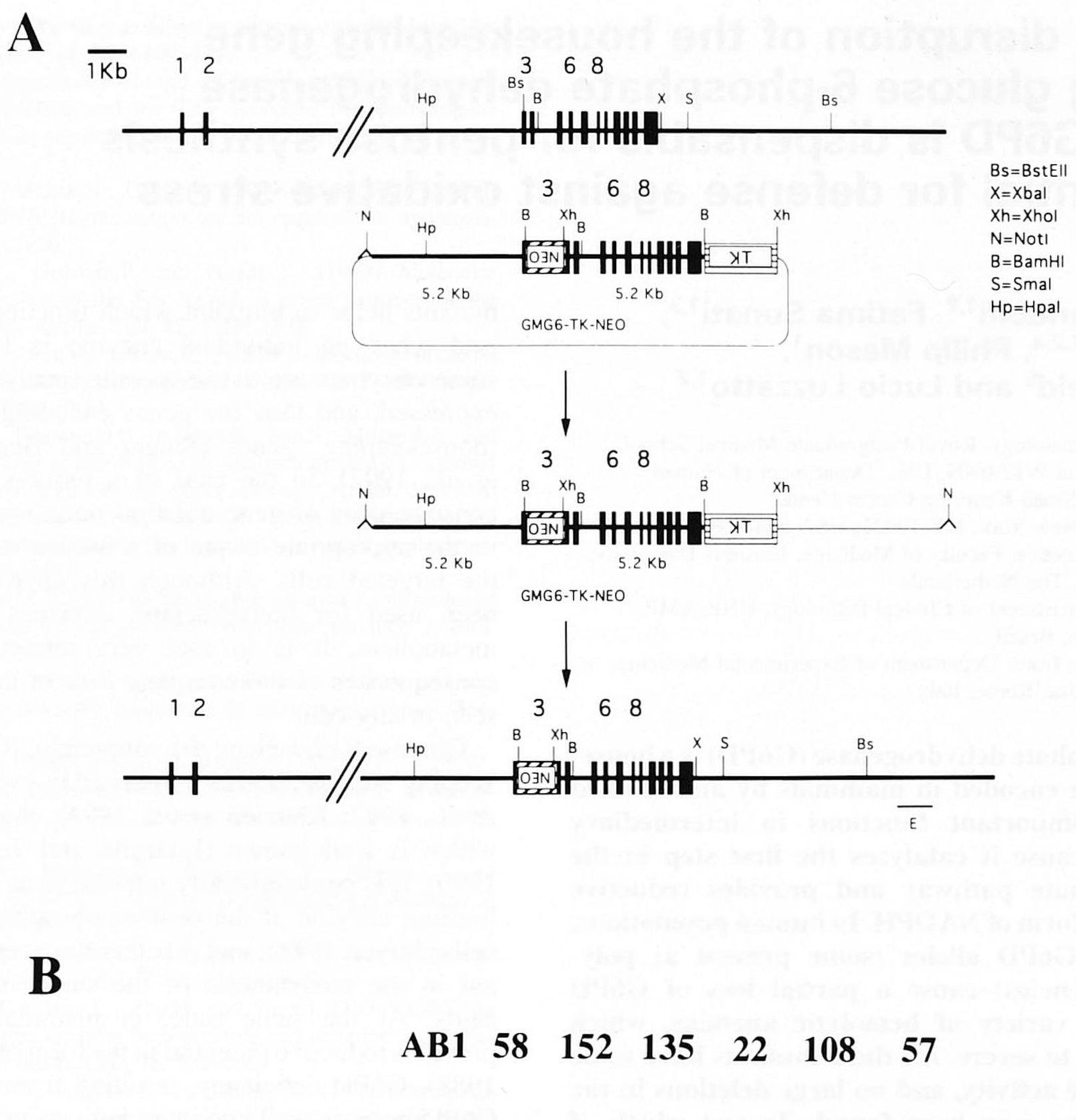

Fig. 1. Targeted disruption of the G6PD gene. (**A**) Construct used for homologous recombination. A partial restriction map of a portion of the murine G6PD gene is shown at the top. Underneath is displayed the replacement-type targeting vector (Mansour *et al.*, 1988) in circular and linear forms. The predicted structure of the targeted allele is shown at the bottom. The restriction enzyme sites are listed. G6PD exons are indicated by black boxes. The neomycin (NEO) and thymidine kinase (TK) cassettes, located in opposite transcriptional orientations with respect to the G6PD gene, are the diagonally and horizontally striped boxes, respectively. The *Eco*RI–*Eco*RI G6PD genomic fragment (probe E) was used as a probe on Southern blots to identify targeted clones. (**B**) Southern blot analysis of individual ES cell clones. AB1, normal genomic mouse DNA (single band of ~18 kb); 58, 22, 57, clones with an intact G6PD gene; 152, 135, 108, clones with a recombined G6PD gene. (**C**) Histochemical stain for G6PD activity. The well on the left contains control cells; the other three wells contain cells grown from clones with recombined G6PD genes.

Results

Targeted disruption of the G6PD gene in mouse embryonic stem (ES) cells

To obtain functional inactivation of the G6PD gene in murine cells, we first isolated genomic clones from an isogenic 129sv phage genomic library. We then constructed a vector for 'positive and negative' selection (Figure 1A; Mansour *et al.*, 1988). The vector was linearized by cleavage of the single *Not*I site, and then transfected by electroporation into AB1 ES cells (McMahon and Bradley, 1990). After 24 h, the cells were subjected to double

Table I. Complete inactivation of G6PD by targeted disruption

ES cell clones	G6PD activity[a] (IU/mg protein)
Non-recombinant[b] (n = 3)	55.5 ± 2.5
Recombinant[c] (n = 3)	not detectable

[a]G6PD activity was measured (Horecker and Smyrniotis, 1955) on cell extracts from culture grown from individual clones for a period of 1 month in the absence of fibroblast feeder layer, in a medium supplemented with 1000 IU/ml leukemia inhibitory factor (Smith *et al.*, 1988; Williams *et al.*, 1988; Nichols *et al.*, 1990).
[b]Clones AB1 (untransfected), and 58 and 178 (not recombined by Southern blot analysis).
[c]Clones 180, 135 and 110 (recombined).

selection with G418 and gancyclovir. A total of 156 individual clones were isolated. Southern blots were hybridized with a probe external to the region of homology (Figure 1A). Non-germline bands were detected in 17 cases (Figure 1B).

Demonstration of the G6PD null phenotype

Individual clones with a rearranged G6PD gene were grown and screened by histochemical staining for G6PD activity (Figure 1C). By this procedure the amount of formazan precipitate (deep purple) depends on the amount of G6PD in the cells. Normal ES cells will be heavily stained. All the clones with a disrupted G6PD gene showed only background staining.

Three clones were picked at random for further characterization. In all three we found no G6PD activity by spectrophotometric assay (Table I). Based on the sensitivity of this technique, we estimate that the recombined cells must have <0.1% of the wild-type G6PD activity. We infer that gene disruption has successfully produced functional inactivation of the G6PD gene.

G6PD null cells are viable but have a reduced cloning efficiency

Having found that G6PD null cells are viable, we proceeded to assess their pattern of growth. We observed that the initial growth rate is normal, but growth becomes significantly impaired as cell density increases (Figure 2), suggesting that some catabolite may limit or inhibit growth. A more stringent test of competence for growth is the capacity of individual cells to form colonies. In this respect, the cloning efficiency of G6PD null cells is decreased dramatically (Figures 3 and 6, histogram on the left). The mean cloning efficiency was 51.2 ± 9.4% for control cells and 5.7 ± 1.8% for G6PD null cells.

G6PD null cells are sensitive to oxidative stress

Because of the known role of G6PD in providing reductive potential in the form of NADPH, we next subjected the cells to agents known to produce oxidative stress. Diamide is a powerful sulfydryl group oxidizing agent. It specifically causes the depletion of glutathione (GSH) in red cells without any other observable effect (Kosower *et al.*, 1969), and nucleated cells that are severely G6PD-deficient are also highly sensitive to diamide (Rosenstraus and Chasin, 1975; D'Urso *et al.*, 1983). Diamide has little effect on the cloning efficiency of normal cells, but it causes a further dose-related reduction in the cloning efficiency of G6PD null cells (Figure 4). Hydrogen peroxide is probably

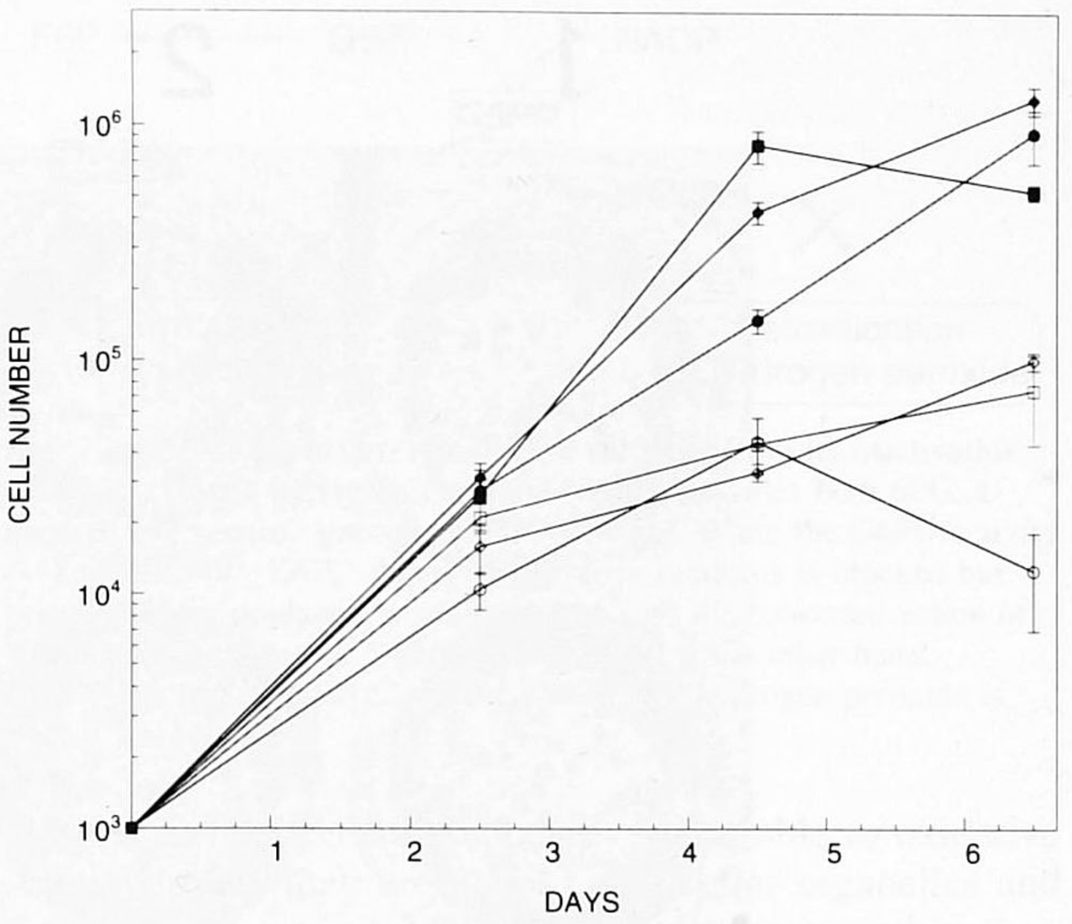

Fig. 2. G6PD null cells have a reduced growth rate at high density. Individual Linbro wells were each inoculated with 1000 ES cells from individual clones. At serial times, cells were harvested by trypsinization and counted in a hemocytometer chamber. Each point was run in triplicate: vertical bars indicate ± one standard deviation. Black symbols represent control clones; white symbols represent G6PD null clones. (●) AB1 untransfected ES cells; (■, ◆) clones transfected but not recombined.

the form of reactive oxygen to which cells are most commonly exposed *in vivo* (Cohen and Hochstein, 1964). Because hydrogen peroxide is very unstable, we have chosen to provide steady-state exposure to this agent by adding glucose oxidase to the culture medium, which will produce hydrogen peroxide from the glucose present in the medium (Moguilevsky *et al.*, 1992). Increasing concentrations of glucose oxidase, not surprisingly, decrease the cloning efficiency of normal cells, but the effect on G6PD null cells is far more drastic (Figure 5). In the intervening 24 h period between changes of medium, the glucose concentration in the presence of glucose oxidase only decreased from 20.5 to 16.1 mM; it is not likely that this change could influence cloning efficiency.

Oxidative stress is responsible for the reduced cloning efficiency of G6PD null cells

The finding that G6PD null cells are hypersensitive to oxidative stress raised the possibility that this might be particularly limiting for their ability to grow into a visible colony, thus explaining their low cloning efficiency. Indeed, when we reduced the partial pressure of oxygen from 22 to 13%, the cloning efficiency was slightly decreased in control cells but significantly increased in G6PD null cells (Figure 6), confirming that their low cloning efficiency may result from oxidative stress.

Discussion

The original purpose of our work was 2-fold: (i) to determine whether the complete absence of G6PD activity was at all compatible with cell viability; and (ii) if so, to determine the main consequences of this major biochemical change on the physiology of the cells.

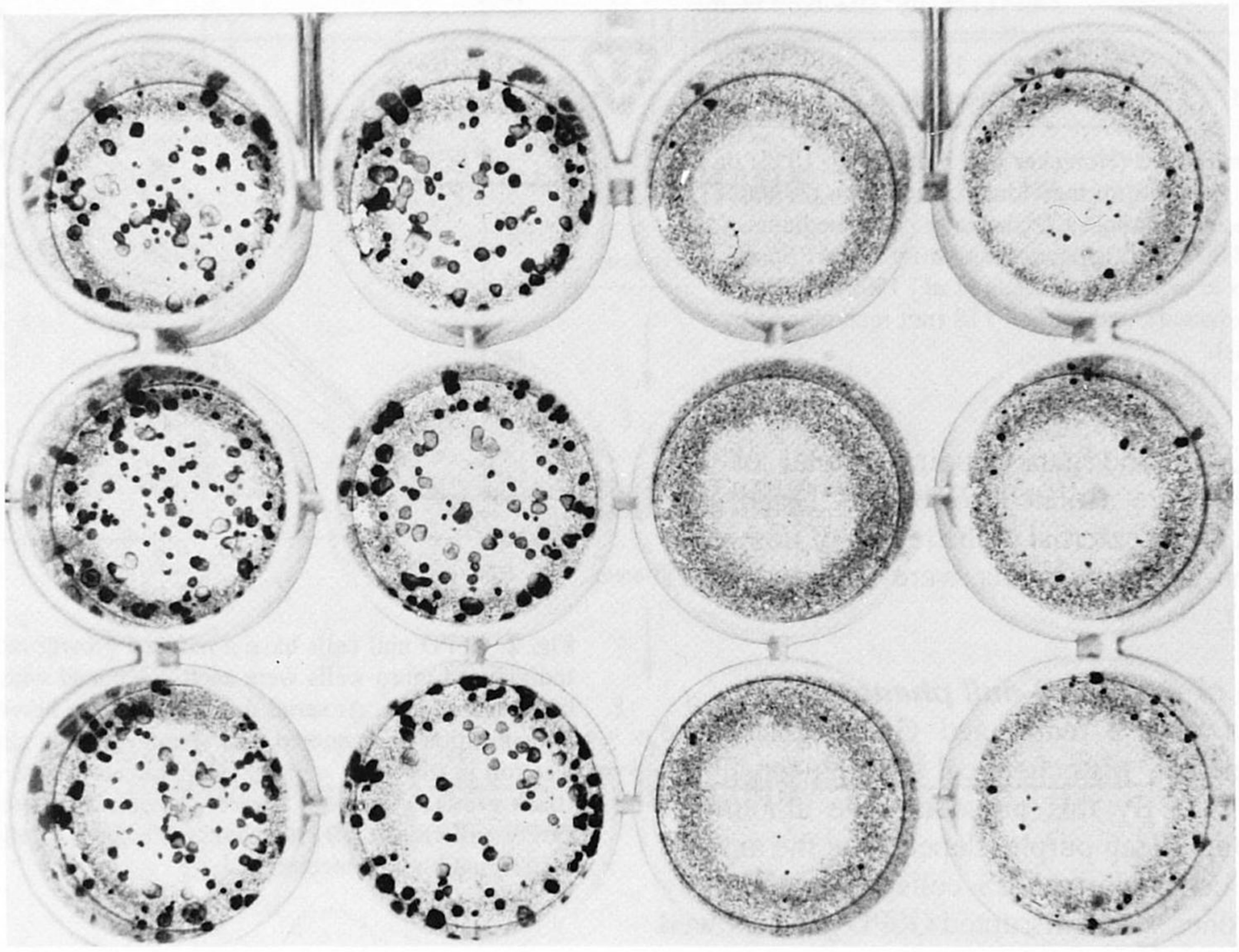

Fig. 3. G6PD null cells have a markedly impaired cloning capacity. Individual wells were inoculated with 100 cells each. After 7 days, the wells were washed, fixed in methanol and stained with Giemsa. Wells in columns 1 and 2 are controls; wells in columns 3 and 4 are G6PD null cells.

G6PD null ES cells

With respect to the first point, our findings prove that G6PD is not indispensable for the survival and growth of ES cells under certain conditions, which we can refer to as basal. To some extent one might regard this result as not too surprising, because G6PD null mutations have been reported previously in bacteria (*Escherichia coli*; Fraenkel, 1968) and yeast (*Saccharomyces cerevisiae*; Lobo and Maitra, 1982; Nogae and Johnston, 1990). In yeast, the mutation makes the cells auxotrophic for methionine by a mechanism that is still obscure (Thomas *et al.*, 1991). However, mammalian cells are more complex, and we cannot predict what would happen when a key enzyme in a major metabolic pathway is ablated. To the best of our knowledge, the only bona fide housekeeping gene for which a 'knock-out' experiment has been reported is hypoxanthine guanine phosphoribosyl transferase (Hooper *et al.*, 1987; Kuehn *et al.*, 1987). Although the expression of this gene is widespread, it functions in a so-called salvage pathway. In mice, its deficiency failed to cause the devastating effects on the central nervous system that it causes in humans. In the case of the glycolytic enzyme glucose 6-phosphate isomerase (GPI), a null mutation has proved lethal in homozygous mice (West, 1993). This mutant was obtained by conventional mutagenesis rather than by gene targeting. The mutation was not analyzed at the molecular level and GPI null cells have not been characterized.

In the case of G6PD, our results show unambiguously that the supply of pentose for nucleic acid synthesis is not an irreplaceable function of this enzyme. In this case, as in many others (Rudnicki *et al.*, 1992; Gorry *et al.*, 1994),

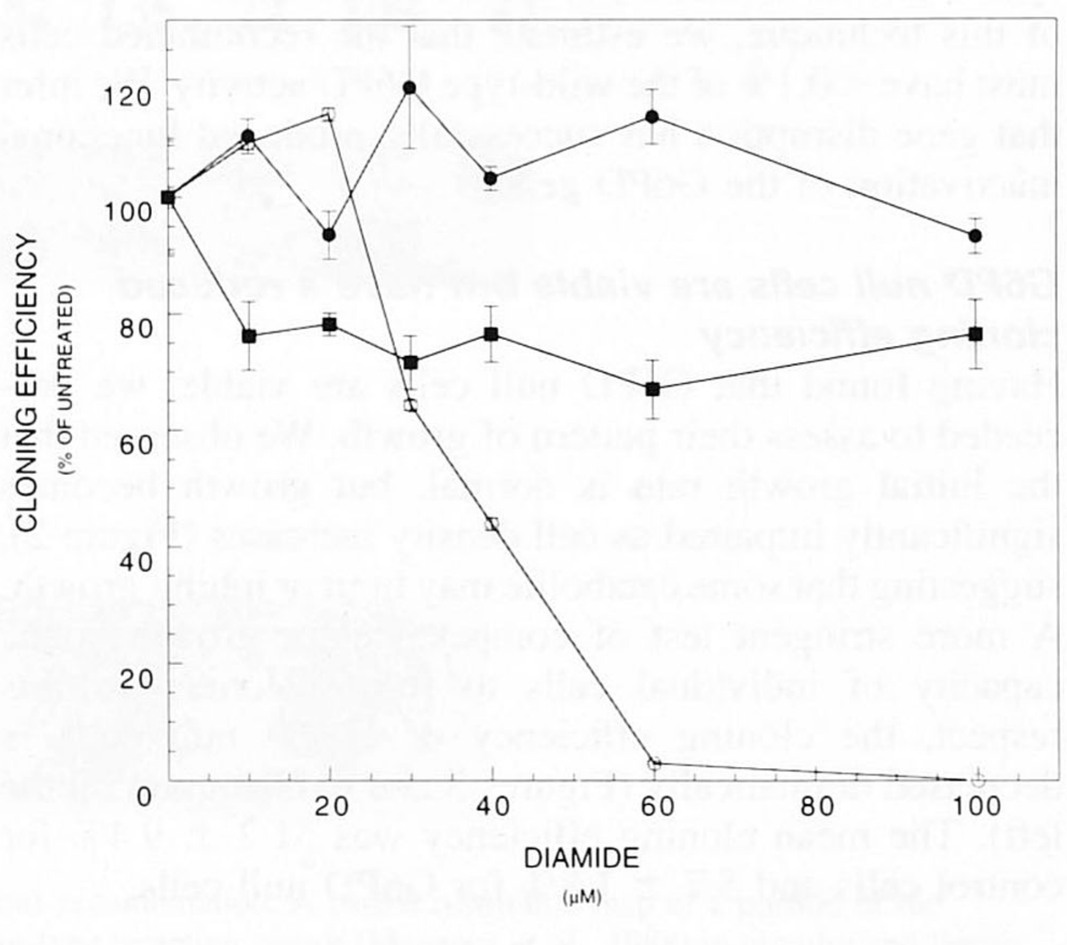

Fig. 4. G6PD null cells are highly sensitive to diamide action. Results are expressed in percent of cloning efficiency obtained in the absence of diamide. Results are the means for AB1 untransfected ES cells (●); two clones transfected but not recombined (■), and three G6PD null clones (○), each run in triplicate. The cloning efficiency in the absence of diamide was 51.2 ± 9.4% for control cells and 5.7 ± 1.8% for G6PD null cells.

there seems to be 'redundancy' in the mammalian cells. We presume that pentose in G6PD null cells is provided entirely by the transketolase–transaldolase (TK–TA) pathway, by-passing G6PD (Figure 7). Of course, this does not mean that, when present, G6PD does not contribute to pentose synthesis. In addition, it is possible that the

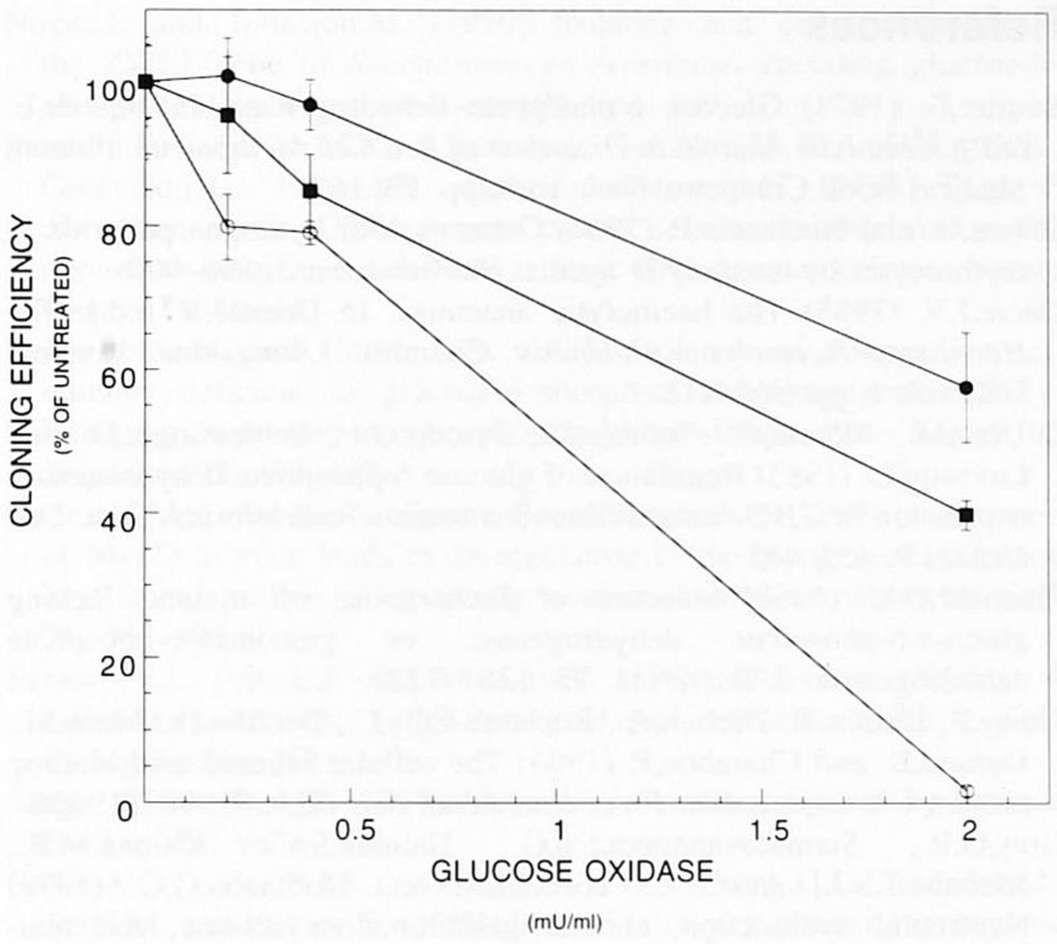

Fig. 5. Hydrogen peroxide challenge abolishes the residual cloning capacity of G6PD null cells. Results are expressed as in Figure 4.

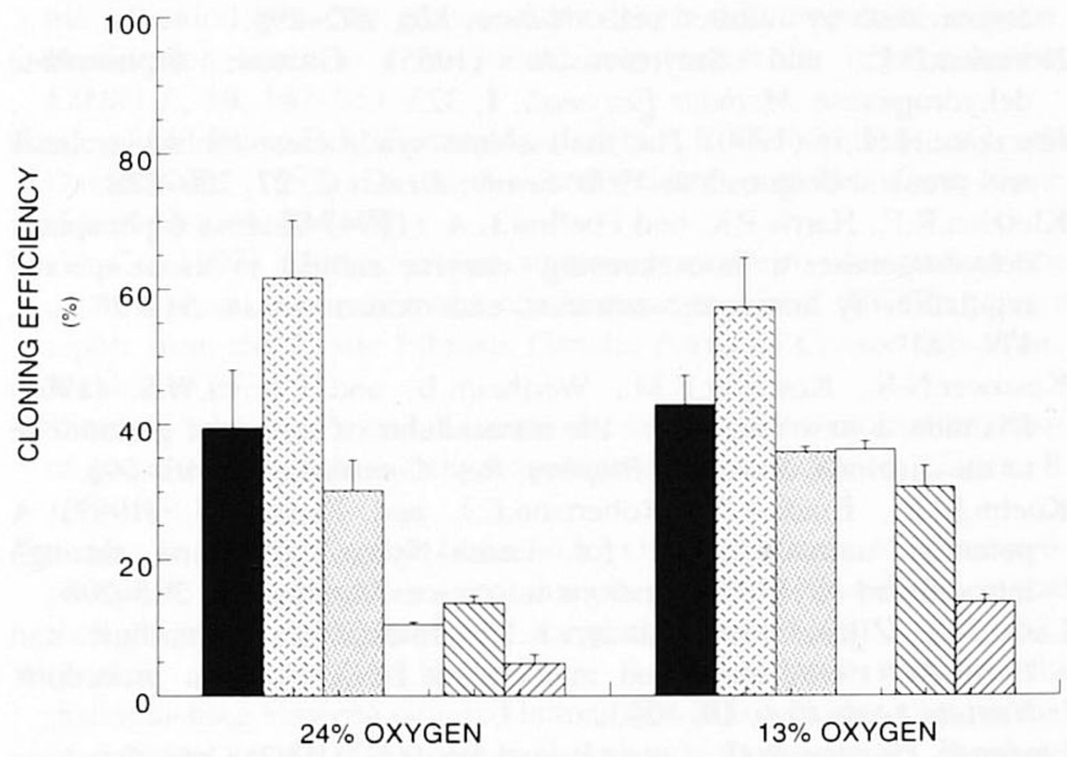

Fig. 6. Oxygen damage is responsible for the low cloning efficiency of G6PD null cells. Cells from three control and three G6PD null clones were inoculated and processed as described in the legend to Figure 3. Left: results for plates incubated in a gas phase consisting of 5% CO_2 in air. Right: results for plates incubated in a vacuum desiccator jar in which O_2 tension had been reduced to 13% by burning a candle. Results are the means of a representative experiment run in triplicate. The darkest bar identifies the AB1 untransfected cells; to its immediate right are two control clones transfected but not recombined (dashed and dotted bars); further to the right are three G6PD null clones (one white and two hatched bars).

TK–TA pathway on its own becomes rate-limiting in conditions different from those we have tested. For instance, in other or more rapidly growing cells, G6PD might be required for an optimal rate of cell growth.

Metabolic consequences of G6PD ablation

With respect to the second point, the evidence accrued from the study of human G6PD deficiency already suggests that G6PD is important in confronting oxidative stress; indeed, it was the clinical problem of drug-induced acute hemolytic anemia that led to the discovery of this condition. This importance can be explained by at least three considerations. (i) Red cells are constantly exposed to oxidative stress because oxygen-free radicals are generated whenever hemoglobin is oxidized (Winterbourn *et al.*,

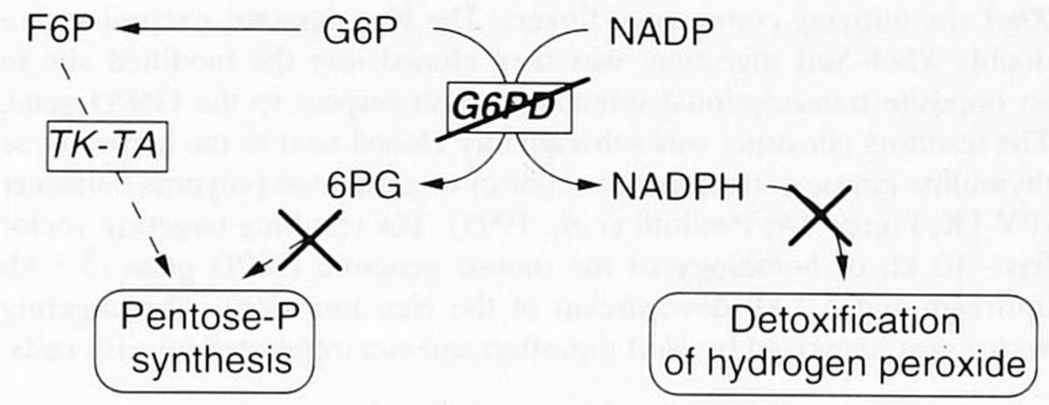

Fig. 7. Metabolic function of G6PD as outlined from its inactivation. Normally, G6PD, acting on G6P and NADP, provides both 6PG, a precursor of pentose phosphate, and NADPH. When the G6PD activity is 'knocked out', G6PD-dependent pentose synthesis is blocked but pentose is still produced, presumably through the concerted action of TK and TA on fructose 6-phosphate (F6P). On the other hand, NADPH is lacking, and thus detoxification of hydrogen peroxide is totally blocked.

1986). (ii) Red cells are specially vulnerable to oxidative stress because they are devoid of cellular organelles and therefore have no NADP-generating enzyme other than G6PD [and 6-phosphogluconate (6PGD), which depends on G6PD for the supply of substrate]. (iii) The level of G6PD is much lower in red cells than in nucleated cells, and G6PD deficiency in nucleated cells of mutant subjects is much less severe than in red cells (Luzzatto and Mehta, 1995). For these reasons, it was not possible to predict whether G6PD might be essential for protection against oxidative injury in cells other than red cells. Our data show quite clearly, by two complementary approaches, that the answer is affirmative. On the one hand diamide, an agent that specifically depletes cellular GSH, is much more toxic for G6PD null cells than for control cells. Interestingly, the same was observed in G6PD null yeast cells (Nogae and Johnston, 1990). On the other hand, decreasing the oxygen partial pressure by ~40% in the gaseous phase of the culture medium restores the cloning efficiency of G6PD null cells to practically normal values. Thus, the low cloning efficiency measured in our first experiments can be attributed not to an unspecific effect of G6PD ablation, but to its specific role *vis-à-vis* oxygen toxicity.

Based on the findings we have reported, it would seem legitimate to regard the defense against oxidative stress as the primary physiological role of G6PD in mammalian cells (Figure 7). One implication of some interest is that other NADPH-generating enzymes, which must be present in ES cells, do not seem to be able to act as surrogates for G6PD: we do not know whether this is because of cellular compartmentalization or simply quantitative insufficiency. The effect of oxygen and of oxygen radicals is so striking that in mammalian cells we can regard the G6PD null mutation as conditionally lethal. It remains to be seen whether it will be such in other cells, or whether at some stage in embryonic development it will be unconditionally lethal.

Materials and methods

Construction of the targeting vector

Murine G6PD clones were isolated from an isogenic 129sv mouse genomic λ library and characterized according to standard procedures (Sambrook *et al.*, 1989). The targeting vector GMG6-TK-NEO was constructed by inserting the pMC1Neo poly(A)$^+$ cassette (Stratagene, La Jolla, CA) into the third exon of the murine G6PD gene. For this purpose, a *Bst*EII site in exon 3 was blunted and transformed into a

*Xho*I site utilizing commercial linkers. The Neo cassette, excised with a double *Xho*I–*Sal*I digestion, was then cloned into the modified site in an opposite transcriptional orientation with respect to the G6PD gene. The resulting construct was subsequently cloned next to the herpes virus thymidine kinase gene under the control of a mutated polyoma enhancer (PY-TK; Figure 1A; Pandolfi *et al.*, 1995). The resulting targeting vector has ~10 kb of homology to the mouse genomic G6PD gene (5.2 kb upstream and 5.2 kb downstream of the Neo insertion). The targeting vector was linearized by *Not*I digestion and electroporated into ES cells.

ES culture and transfection

AB1 ES cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 15% fetal calf serum (FCS) on SNL feeder (McMahon and Bradley, 1990) and transfected as follows. 10^7 cells were resuspended in Hank's balanced salt solution (HyClone, Logan, UT) with or without (mock experiment) 30 μg of linearized plasmid, and subjected to a double shock (240 and 260 V at 960 μF). The selection was started 24 h later in a medium containing 350 μg/ml G418 and 2 mM gancyclovir. After 8 days of selection, 156 individual clones, out of >200 double-resistant clones, were picked and grown individually.

Analysis of double-resistant ES cell clones

Genomic DNA from 129 sv mouse, wild-type AB1 ES cells and prospective targeted ES clones was isolated as described previously (Laird *et al.*, 1991). It was digested with *Hpa*I and *Xho*I restriction enzymes and fractionated by electrophoresis on 0.5 or 0.6% agarose gels. Genomic DNA was subsequently transferred by alkaline capillary blotting to positively charged nylon membranes and hybridized to an *Eco*RI–*Eco*RI 3′ G6PD genomic DNA fragment external to the targeting vector (probe E; Figure 1A). An 18 kb band is detected if the murine G6PD gene is in the wild-type configuration. Because of a *Xho*I site present within the Neo cassette, a smaller band of 15 kb is detected if the gene is targeted. A total of 17 clones out of 156 showed a restriction fragment compatible with recombination (11%).

Histochemical analysis and quantitation of G6PD

Subconfluent ES cells in 24-well plates were stained according to a modified version of the method described by Wajntal and De Mars (1967). This method takes advantage of the fact that the NADPH produced by the G6PD reaction, in the presence of the electron carrier phenazine methosulfate, reduces a soluble tetrazolium dye to insoluble formazan. The cells were washed twice with PBS, then drained dry for ~30 min at room temperature and placed in staining solution (10 mM G6P, 0.2 mM NADP, 0.5 mM $MgCl_2$, 12 mM NaN_3, 0.5 mg/ml Nitro BT, 0.02 mg/ml phenazine in PBS/H_2O, 4:1) at 37°C in the dark for 40 min. The cells were rinsed in PBS, mounted in glycerol and observed immediately. G6PD activity on cell extracts was measured as described previously (Horecker and Smyrniotis, 1955).

ES cell colony forming assay in the presence of diamide, H_2O_2 and at reduced O_2 tension

The colony forming assays were carried out by plating 100 ES cells per well in a 24-well plate. Each clone was plated in triplicate. Each experiment was performed twice. The ES cells were resuspended in DMEM, 15% FCS on SNL feeder in the absence and presence of diamide (Sigma, St Louis, MO) at final concentrations of 10, 20, 30, 40, 60 and 100 μM. The diamide medium was replaced daily. After 7 days, the clones were washed three times with PBS, stained with May Grünwald–Giemsa and counted.

The colony forming assays were carried out in the presence or absence of glucose oxidase (EC 1.1.3.4, Sigma) as a source of H_2O_2, at final concentrations of 0.2, 0.4 and 2.0 mIU/ml. The medium was changed daily. After 7 days the colonies were counted as described previously.

To reduce O_2 tension, the plates were incubated in a vacuum desiccator jar in which O_2 tension had been reduced to 13% by burning a candle. Control plates were incubated in a gas phase consisting of 5% CO_2 in air.

Acknowledgements

We thank S.Nicolis and R.Lovell-Badge for the 129 genomic library, A.Bradley for the AB1 ES cells, C.Corcoran for assistance with the quantitative assay of G6PD activity and discussions, T.Vulliamy for helpful comments and P.G.Pelicci for critical reading of the manuscript.

References

Beutler,E. (1978) Glucose 6-phosphate dehydrogenase. In Beutler,E. (ed.), *Hemolytic Anemia in Disorders of Red Cell Metabolism*. Plenum Medical Book Company, New York, pp. 23–167.

Cohen,G. and Hochstein,P. (1964) Generation of hydrogen peroxide in erythrocytes by haemolytic agents. *Biochemistry*, **3**, 895–900.

Dacie,J.V. (1985) The haemolytic anaemias. In Dacie,J.V. (ed.), *The Hereditary Haemolytic Anaemias*. Churchill Livingstone, London, UK, Vol. 1, pp. 364–418.

D'Urso,M., Mareni,C., Toniolo,D., Piscopo,M., Schlessinger,D. and Luzzatto,L. (1983) Regulation of glucose 6-phosphate dehydrogenase expression in CHO–human fibroblast somatic cell hybrids. *Som. Cell Genet.*, **9**, 429–443.

Fraenkel,D.G. (1968) Selection of *Escherichia coli* mutants lacking glucose-6-phosphate dehydrogenase or gluconate-6-phosphate dehydrogenase. *J. Bacteriol.*, **95**, 1267–1271.

Gorry,P., Lufkin,T., Dierich,A., Rochette-Egly,C., Decimo,D., Mark,M., Durand,B. and Chambon,P. (1994) The cellular retinoic acid binding protein I is dispensable. *Proc. Natl Acad. Sci. USA*, **91**, 9032–9036.

Gray,G.R., Stamatoyannopoulos,G., Naiman,S.C., Kliman,M.R., Klebanoff,S.J., Austin,T., Yoshida,A. and Robinson,G.C. (1973) Neutrophil dysfunction, chronic granulomatous disease, and non-spherocytic haemolytic anaemia caused by complete deficiency of glucose-6-phosphate dehydrogenase. *Lancet*, **2**, 530–534.

Hooper,M., Hardy,K., Handyside,A., Hunter,S. and Monk,M. (1987) HPRT-deficient (Lesch–Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature*, **326**, 292–295.

Horecker,B.L. and Smyrniotis,A. (1955) Glucose 6-phosphate dehydrogenase. *Methods Enzymol.*, **1**, 323.

Kazazian,H.H.,Jr (1990) The thalassemia syndromes: molecular basis and prenatal diagnosis in 1990. *Semin. Hematol.*, **27**, 209–228.

Kletzien,R.F., Harris,P.K. and Foellmi,L.A. (1994) Glucose 6-phosphate dehydrogenase: a 'housekeeping' enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. *FASEB J.*, **8**, 174–181.

Kosower,N.S., Kosower,E.M., Wertheim,B. and Correa,W.S. (1969) Diamide, a new reagent for the intracellular oxidation of glutathione to the disulfide. *Biochem. Biophys. Res. Commun.*, **37**, 593–596.

Kuehn,M.R., Bradley,A., Robertson,E.J. and Evans,M.J. (1987) A potential animal model for Lesch–Nyhan syndrome through introduction of HPRT mutations into mice. *Nature*, **326**, 295–298.

Laird,P.W., Zijderveld,A., Linders,K., Rudnicki,M.A., Jaenisch,R. and Berns,A. (1991) Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.*, **19**, 4293.

Larsen,F., Gundersen,G., Lopez,R. and Prydz,H. (1992) CpG islands as gene markers in the human genome. *Genomics*, **13**, 1095–1107.

Levy,H.R. (1979) Glucose-6-phosphate deydrogenase. *Adv. Enzymol.*, **48**, 97–192.

Lobo,Z. and Maitra,P.K. (1982) Pentose phosphate pathway mutants of yeast. *Mol. Gen. Genet.*, **185**, 367–368.

Luzzatto,L. (1993) Glucose 6-phosphate dehydrogenase deficiency and hemolytic anemia. In Nathan,D.G. and Oski,D.F. (eds), *Hematology of Infancy and Childhood*. W.B.Saunders, Philadelphia, PA, pp. 674–691.

Luzzatto,L. and Mehta,A. (1995) Glucose 6-phosphate dehydrogenase deficiency. In Scriver,C.R., Beaudet,A.L., Sly,W.S. and Valle,D. (eds), *The Metabolic Basis of Inherited Diseases*. 7th edn. McGraw-Hill, New York, pp. 3367–3398.

Luzzatto,L. and Testa,U. (1978) Human erythrocyte glucose 6-phosphate dehydrogenase: structure and function in normal and mutant subjects. *Curr. Top. Hematol.*, **1**, 1–70.

Mansour,S.L., Thomas,K.R. and Capecchi,M.R. (1988) Disruption of the proto-oncogene int in mouse embryo-derived stem cells: a general strategy for mutations to non-selectable genes. *Nature*, **366**, 348–352.

McMahon,A.P. and Bradley,A. (1990) The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell*, **62**, 1073–1085.

Moguilevsky,N., Steens,M., Thiriart,C., Prieels,J.P., Thiry,L. and Bollen,A. (1992) Lethal oxidative damage to human immunodeficiency virus by human recombinant myeloperoxidase. *FEBS Lett.*, **302**, 209–212.

Monaco,A.P. (1993) Molecular human genetics and the Duchenne/Becker muscular dystrophy gene. *Mol. Cell. Biol. Hum. Diseases Ser.*, **3**, 1–11.

Nichols,J., Evans,E.P. and Smith,A.G. (1990) Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity. *Development*, **110**, 1341–1348.

Nogae,I. and Johnston,M. (1990) Isolation and characterization of the ZWF1 gene of *Saccharomyces cerevisiae*, encoding glucose-6-phosphate dehydrogenase. *Gene*, **96**, 161–169.

Pandolfi,P.P., Roth,M.E., Karis,A., Leonard,M.W., Dzierzak,E., Grosveld,F.G., Engel,J.D. and Lindenbaum,M.H. (1995) Targeted disruption of the GATA-3 gene causes severe abnormalities in the nervous system and a failure in fetal liver haematopoiesis. *Nature Genet.*, **11**, 40–44.

Rosenstraus,M. and Chasin,L.A. (1975) Isolation of mammalian cell mutants deficient in glucose-6-phosphate dehydrogenase activity: linkage to hypoxanthine phosphoribosyl transferase. *Proc. Natl Acad. Sci. USA*, **72**, 493–497.

Rudnicki,M.A., Braun,T., Hinuma,S. and Jaenisch,R. (1992) Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell*, **71**, 383–390.

Sambrook,J., Fritz,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Singer,M. and Berg,P. (1991) *Genes and Genomes*. University Science Books, Mill Valley, CA.

Smith,A.G., Heath,J.K., Donaldson,D.D., Wong,G.G., Moreau,J., Stahl,M. and Rogers,D. (1988) Inhibition of pluripotential embryonic stem cell differentiation purified polypeptides. *Nature*, **336**, 688–690.

Stryer,L. (1989) *Biochemistry*. 3rd edn. W.H.Freeman and Co., San Francisco, CA.

Thomas,D., Cherest,H. and Surdin-Kerjan,Y. (1991) Identification of the structural gene for glucose-6-phosphate dehydrogenase in yeast. Inactivation leads to a nutritional requirement for organic sulfur. *EMBO J.*, **10**, 547–553.

Tinsley,J.M., Blake,D.J., Pearce,M., Knight,A.E., Kendrick-Jones,J. and Davies,K.E. (1993) Dystrophin and related proteins. *Curr. Opin. Genet. Dev.*, **3**, 484–490.

Tsui,L.C. (1992) Mutations and sequence variations detected in the cystic fibrosis transmembrane conductance regulator (CFTR) gene: a report from the Cystic Fibrosis Genetic Analysis Consortium. *Hum. Mutat.*, **1**, 197–203.

Vulliamy,T., Mason,P. and Luzzatto,L. (1992) The molecular basis of glucose-6-phosphate dehydrogenase deficiency. *Trends Genet.*, **8**, 138–143.

Vulliamy,T., Beutler,E. and Luzzatto,L. (1993) Variants of glucose-6-phosphate dehydrogenase are due to missense mutations spread throughout the coding region of the gene. *Hum. Mutat.*, **2**, 159–167.

Wajntal,A. and DeMars,R. (1967) A tetrazolium method for distinguishing between cultured human fibroblasts having eiter normal or deficient levels of glucose-6-phosphate dehydrogenase. *Biochem. Gen.*, **1**, 61–64.

West,J.D. (1993) A genetically defined animal model of anembryonic pregnancy. *Hum. Reprod.*, **8**, 1316–1323.

Williams,R.L. *et al.* (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature*, **336**, 684–687.

Winterbourn,C.C., Benatti,U. and De Flora,A. (1986) Contributions of superoxide, hydrogen peroxide and transition metal ions to auto-oxidation of the favism-inducing pyrimidine aglycone, divicine, and its reactions with haemoglobin. *Biochem. Pharmacol.*, **35**, 2009–2015.

Received on May 4, 1995; revised on July 24, 1995