Overexpression of liver-type phosphofructokinase (PFKL) in transgenic-PFKL mice: implication for gene dosage in trisomy 21

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The human liver-type subunit of the key glycolytic enzyme, phosphofructokinase (PFKL), is encoded by a gene residing on chromosome 21. This chromosome, when triplicated, causes the phenotypic expression of Down's syndrome (trisomy 21). Increased phosphofructokinase activity, a result of gene dosage, is commonly found in erythrocytes and fibroblasts from Down's syndrome patients. We describe the construction of transgenic mice overexpressing PFKL for use as a well-defined model system, in which the effects of PFKL overexpression in various tissues, and throughout development, can be studied. Mice transgenic for a murine PFKL 'gene cDNA' hybrid construct were found to overexpress PFKL in a tissue-specific manner resembling that of the endogenous enzyme. Although unchanged in adult brain, PFK specific activity was found to have been almost doubled in brains of embryonic transgenic-PFKL mice, suggesting that the extra copies of the PFKL gene are expressed during the developmental period. This pattern of overexpression of PFKL in brains of transgenic-PFKL mice suggests that gene-dosage effects may be temporally separated from some of their consequences, adding an additional layer of complexity to the analysis of gene dosage in trisomy 21.

INTRODUCTION

Down's syndrome (DS or trisomy 21) is a human genetic abnormality caused by triplication of the distal half of the large arm of chromosome 21, a region cytologically known as band 21q22 (Summit, 1981; Korenberg, 1990). DS patients characteristically suffer from a wide variety of structural and functional abnormalities which include: mental retardation, morphogenic abnormalities, a wide range of metabolic and endocrinological aberrations, defects in the immune system and the development of Alzheimer's-type pathology in the brains of patients who survive into their forties and beyond (Tolksdorf and Wiedemann, 1981; Epstein, 1986).

The basic mechanism that causes DS is believed to be the existence of proportionality between the copy number of a gene and the amount of its product, known as a gene-dosage effect. It is believed that the added copies of genes present in the triplicated chromosomal fragment are generally expressed in a manner similar to the expression of the genes present in the normal complement of DNA, giving rise to added amounts of their products; these, in turn, are thought to upset the balance of biochemical reactions and other processes that ensure the normal functioning of tissues and organs (Epstein et al., 1981; Epstein and Epstein, 1981; Epstein, 1986). Yet, the precise connections between overexpression of specific genes from the 21q22 region and specific symptoms of the disease are still largely unknown (Groner and Elson, 1991). It is believed that the DS phenotype is the result of combined effects of overexpression of several genes, or of groups of genes, on chromosome 21 rather than overexpression of a single 'DS gene'.

One of the genes mapped to band 21q22 is that of the liver-type subunit of the key glycolytic enzyme phosphofructokinase (PFK), which catalyses the transfer of a phosphate group from ATP to fructose 6-phosphate (Vora and Francke, 1981; Cox et al., 1984; Van Keuren et al., 1986). This reaction is considered to be a major rate-limiting step of glycolysis, and PFK activity is extensively regulated by a wide variety of positive and negative effectors [reviewed in Uyeda (1979) and Dunaway (1983)]. The catalytically active form of PFK is a tetramer, which can be composed of any combination of three types of subunits termed M, P (or C) and L for muscle, platelet and liver type respectively. Each subunit is encoded by a separate gene; only that of PFKL maps to chromosome 21. In various tissues the three subunit types randomly associate to form catalytically active homo- or hetero-tetramers, the composition of which reflects the relative proportions of each subunit type present and which varies from tissue to tissue and in each tissue throughout development (Vora, 1982; Dunaway et al., 1988, 1990; Dunaway and Kasten, 1988, 1989).

The possible connections between PFKL overexpression and DS stem from the mapping of the PFKL gene to the 'DS region' of chromosome 21, and from the findings that the specific activity of PFK is increased by 30–60% in erythrocytes [summarized in Epstein (1986)] and fibroblasts (Anneren et al., 1987) obtained from DS patients. The tetramer profile of erythrocyte PFK has been shown to be abnormal in DS in that the relative amounts of PFKL-containing tetramers were found to be increased at the expense of those which did not, probably as the result of the presence of added amounts of the PFKL subunit and of the random manner by which PFK subunits tetramerize (Vora and Francke, 1981). In addition, several abnormalities in the concentrations of glycolytic intermediates and products as well as in glycolytic rates have previously been described in DS [summarized in Elson et al. (1992)], suggesting that PFKL overexpression might be a cause of some of the abnormalities present in DS. However, simultaneous overexpression of other genes from the DS region in tissue samples from DS patients makes the analysis of the possible effects of PFKL overexpression in the disease extremely complicated.

Abbreviations used: PFK, phosphofructokinase; PFKL, liver-type PFK; DS, Down's syndrome; Tg-PFKL mice, mice transgenic for murine PFKL; 1 × SSC, 0.15 M NaCl + 0.015 M sodium citrate.

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We have attempted to isolate and analyse the effects of PFKL overexpression by constructing model systems where PFKL is the only gene from the DS region to be overexpressed. We previously described rat PC12 cells which were stably transfected with the human PFKL cDNA and in which *in vitro* PFK activity was increased by 40–60 % (Elson et al., 1992). PC12-PFKL cells performed glycolysis 40 % faster than controls, and PFK activity isolated from these cells was found to have altered biochemical-regulatory properties. The latter phenomenon was found also in fibroblasts from DS patients, but not in the erythrocytes (Elson et al., 1992). The amount of protein kinase C in PC12-PFKL cells was found to have been increased by 70–80 %, with most of the additional protein kinase C found in the activated membrane-bound form of this enzyme. mRNA levels of Jun B, C-Jun and Jun D were found to be elevated in these cells as well (A. Elson, D. Levanon, Y. Weiss and Y. Groner, unpublished work). These findings suggest that the results of PFKL overexpression might be further propagated through the ability of protein kinase C and Jun to affect expression of other genes, resulting in relatively widespread alterations in gene expression.

The present work describes the construction of mice transgenic for murine PFKL (Tg-PFKL mice) for use as a model system in determining the physiological consequences of PFKL overexpression.

EXPERIMENTAL

Construction of the mouse PFKL mini-gene used for microinjection

For generating the murine PFKL mini-gene, genomic fragments from the 5′ and 3′ ends of the murine PFKL gene, as well as its full-length cDNA, were isolated using the human PFKL cDNA as a probe (Levanon et al., 1989). Identification of the cloned fragments originating from the gene for the liver-type subunit of PFK was verified by comparing their sequence with that of the murine PFKL cDNA (Gehnrich et al., 1988). To generate the mini-PFKL construct, the following steps, dictated by the availability of appropriate restriction sites, were implemented. First, genomic sequences of the second exon were ligated to cDNA sequences: a ~ 100 bp genomic *Sac*I–*Bsm*I fragment, in which the *Bsm*I site is located in exon 2, was connected to a *Bsm*I–*Cla*I fragment containing ~ 400 bp of cDNA via the second half of the same *Bsm*I site, generating a construct which we designated fragment 'A'. Next, a genomic *Sac*I fragment of ~ 7 kb was ligated to the *Sac*I site of fragment 'A' to generate the fragment designated 'B'. Hence, fragment 'B' included 2.9 kb upstream of the first exon, followed by the first exon, the first intron and the 400 bp of cDNA sequences. In parallel, the 3′ region of the PFKL cDNA was replaced with genomic fragment; first the cDNA was cleaved at a unique *Nhe*I site in the 3′-untranslated region, and cDNA sequences downstream of this point were substituted with a 1.4 kb *Nhe*I–*Hin*dIII genomic fragment containing the 3′ non-coding region and 1 kb beyond. From this modified cDNA construct, a *Cla*I–*Hin*dIII fragment was isolated (fragment 'C'), and ligated to the previously constructed genomic cDNA hybrid fragment 'B', thus generating the complete mouse PFKL mini-gene as depicted in Figure 1. All the above steps were accompanied by sequence analyses to verify proper ligation of cloned fragments.

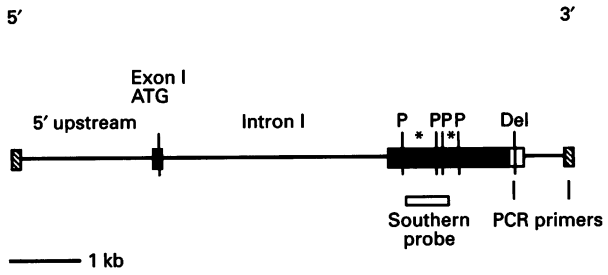

Figure 1 Structure of the murine PFKL mini-gene used for microinjection into fertilized mouse eggs

Heavily drawn regions are coding sequences, the unshaded box represents the 3′ untranslated sequences and thin lines represent genomic or non-coding DNA sequences. The hatched boxes at both ends of the microinjection construct represent vector (Bluescript) sequences. The empty box underneath the construct represents the probe used for Southern-blot hybridization, and asterisks mark the diagnostic fragments that hybridize to the probe; also indicated are the approximate locations of the two primers used for PCR amplification. ATG, initiator ATG; P, *Pvu*II sites relevant for Southern-blot analysis; Del, location of the 22 bp deletion used for identification of transgene-derived mRNA. The size of the entire construct is approximately 10.0 kb.

The 22 bp deletion in the 3′ untranslated region of the mini-gene (Figure 1) was introduced by cleaving the *Cla*I–*Hin*dIII cDNA genomic hybrid fragment 'C' at a unique *Nhe*I site, followed by a treatment with S1 nuclease. Again, the extent of the deletion was determined by DNA sequencing. The 22 bp deletion, 8 bp upstream and 14 bp downstream of the *Nhe*I site, was the smallest deletion found and subsequently used. The mutated fragment replaced fragment 'C' in the final ligation step described above to generate the mutated version of the mini-gene. The DNA to be injected was freed from vector (Bluescript) sequences by digestion with *Bss*HII; the linearized DNA was then purified by agarose-gel electrophoresis, the agarose was removed by adsorbing the DNA to glass powder (Glassmilk; Bio-Rad), the DNA was precipitated with ethanol and resuspended at 10 μg/ml in injection buffer (150 mM KCl, 5 mM NaCl, 10 mM Pipes, pH 7.0).

Transgenic animals

Transgenic mice were produced as previously documented (Epstein et al., 1987; Avraham et al., 1988) by pronuclear microinjection of fertilized oocytes, as described in Hogan et al. (1986). Fertilized eggs were obtained from CYB/6 donors which were synchronized, superovulated by injections of pregnant mare's serum and human chorionic gonadotropin (Sigma), and mated with B6D/2 males. DNA (1–2 pmol) was injected into one pronucleus of each egg, and those that survived injection were implanted into the oviducts of pseudopregnant CD1 females. Pups were born 19–20 days later and were weaned at the age of 3 weeks. The constructs used for microinjection were described above.

DNA preparation and Southern-blot analysis

Genomic DNA was prepared from tail tissue or from one-quarter to one-half of whole embryos using proteinase K, as described in Ausubel et al. (1990). For Southern-blot analysis, 20 μg of genomic DNA was digested with *Pvu*II and blot-hybridized with a 1227 bp-long *Stu*I fragment of the mouse PFKL cDNA (positions 271–1498, Figure 1), which had been labelled with ^{32}P. This combination of probe and restriction endonuclease resulted in the appearance of two bands diagnostic of the transgene, as described in the Results section.

RNA analysis

Northern blots were prepared from 10 μg of total RNA prepared from mouse tissues, by the LiCl/urea method (Ausubel et al., 1990). The transgene-specific probe used was a 35 bp-long oligomer of the sequence 5′-TTGAGTTCCCCTCGG/TGCTGGGGTGAGAGTCCGGG-3′ (oligo 4113). The sequence of the probe was identical with that of the mutated microinjection construct; the slash denotes the site where 22 bp were deleted so as to mark the transgene specifically. The oligomer was labelled at its 3′ end by terminal transferase. Hybridization conditions were as described in Ausubel et al. (1990); blots were washed in 3 × SSC at 50 °C and exposed. The 3′ untranslated probe was a 327 bp-long fragment starting at position 2255 of the murine PFKL cDNA (Gehnrich et al., 1988), and contained the last 86 bp of the translated region followed by 241 bp from the 3′ untranslated region. Owing to differences in sequence between the PFK subunit genes, the short stretch of nucleotides from the translated region was incapable of recognizing mouse PFKM mRNA on Northern blots.

Analysis of PFK activity in mouse tissues

Erythrocytes

Blood (50 μl) was collected from hearts of mice immediately after death and diluted into 1 ml of 0.9 % NaCl containing 125 units of heparin. The blood suspension was gently centrifuged, and the supernatant and buffy coat layer were aspirated. The erythrocytes were washed twice more in a similar manner with 0.9 % NaCl (without heparin) and lysed in 0.2–0.3 ml of lysis buffer (20 mM Tris/HCl, pH 7.5, 10 mM NaF, 10 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 100 mM EDTA, 10 mM dithiothreitol, 0.1 mM fructose 6-phosphate, 0.2 mM ATP, 0.5 % Nonidet P40, 0.07 unit/ml aprotonin, 0.2 mg/ml phenylmethanesulphonyl fluoride and 25 μg/ml leupeptin). The lysed erythrocyte suspension was centrifuged at 10000 *g*, 4 °C, and the supernatant used for PFK activity measurement.

Other tissues

Liver, brain, heart or kidney samples were removed from freshly killed mice and homogenized in 2–5 ml of lysis buffer. Heart samples were washed in water to remove blood before homogenization; livers were not perfused, and the haemoglobin concentration of liver homogenates, indicating the amount of contaminating blood present, was negligible. Homogenates were spun at 10000 *g* for 15 min at 4 °C, and the supernatant was used for PFK activity determination.

Total PFK activity was measured at pH 8.0 using the microassay technique described in Elson et al. (1992). Blood PFK activity was normalized to haemoglobin content of the lysate, as measured by the ferricyanide–cyanide method (Mattenheimer, 1970). No differences were found in the haemoglobin content of erythrocytes of Tg-PFKL and control mice. PFK activity in other tissues was normalized to the protein content of the samples, determined as described by Bradford (1976), using BSA as a standard.

RESULTS

The construct used for production of transgenic mice was a murine PFKL mini-gene which included cDNA and genomic sequences (see the Experimental section). The decision to use a mini-gene was influenced by observations that constructs based only on cDNA sequences tended to be expressed incorrectly in transgenic mice (Palmiter et al., 1991) and by the relatively large size of the mouse PFKL gene, at least 21.5 kb (Rongnoparut et al., 1991), which created practical difficulties in building a genomic expression construct.

The construction of the PFKL mini-gene is described in the Experimental section and depicted in Figure 1. The mini-gene included 2.9 kb of 5′ genomic regions, followed by the first exon, the entire first intron, and some of the second exon. It was in this exon that genomic sequences were replaced by cDNA sequences which extended on to the 3′ untranslated region; at the end of this region another genomic fragment, which included 700 bp beyond the polyadenylation signal, was attached. This last exchange was performed in order to include genomic sequences from a region where signals important for processing of transcripts are usually found, and to omit the polyadenylated tail. A 22 bp deletion was introduced into the 3′ untranslated region of the mini-gene in order to enable detection of transgene-derived mRNA in the presence of the otherwise identical endogenous PFKL mRNA. This mutation was introduced at a point not known to be necessary for production of the PFKL mRNA.

Human HeLa and mouse embryonic stem cells, stably transfected with either the mutated or non-mutated forms of the mini-gene construct, contained transgene mRNA of the same size as the endogenous PFKL message and exhibited increases of 50–150 % in PFK specific activity. These findings indicated that the transgene was capable of being expressed in cultured cells and of generating authentic enzymically active PFKL (not shown).

Mice transgenic for the PFKL mini-gene construct were generated in a series of pronuclear microinjection experiments, using either the mutated or the non-mutated forms of the construct. Generation of lines transgenic for the non-mutated construct was thought to be a necessary control for the possibility that the mutation introduced into the 3′ untranslated region of the construct would somehow alter its expression. Pups originating in microinjected oocytes were screened for the presence of transgenics by Southern-blot analysis of tail genomic DNA digested with *Pvu*II. The probe used in these analyses was a restriction fragment from the coding region of the murine PFKL cDNA (Figure 1), which hybridized with the mini-gene construct as well as with fragments of the endogenous PFKL gene and possibly, also, with fragments of the genes for PFKM or PFKP. Owing to differences between the restriction maps of the mini-gene and the endogenous PFK genes, the presence of the mini-gene in DNA samples analysed in this manner resulted in the appearance of two diagnostic bands, 997 bp and 269 bp in size. The smaller fragment yielded a weak signal in this analysis because of its short overlap with the probe and was detected only when large amounts of transgene DNA were present (Figure 2). Alternatively, transgenic mice were identified by performing PCR on genomic DNA samples using a primer that was complementary to Bluescript sequences left at the 3′ end of the construct in conjunction with a primer from the 3′ untranslated region of the murine PFKL cDNA (not shown).

A total of 13 independent founder mice were generated; 11 of these carried the mutated form of the transgene, and two contained the non-mutated form. The copy number of each founder mouse was roughly estimated from the intensity of the 997 bp-long diagnostic band in genomic Southern-blot analyses of the type shown in Figure 2. Copy numbers were found to range from 1 to 2 in founders 116, 126, 186 and 201 to several dozen in founders 143 and 224 (Figure 2 and Table 1). Most of

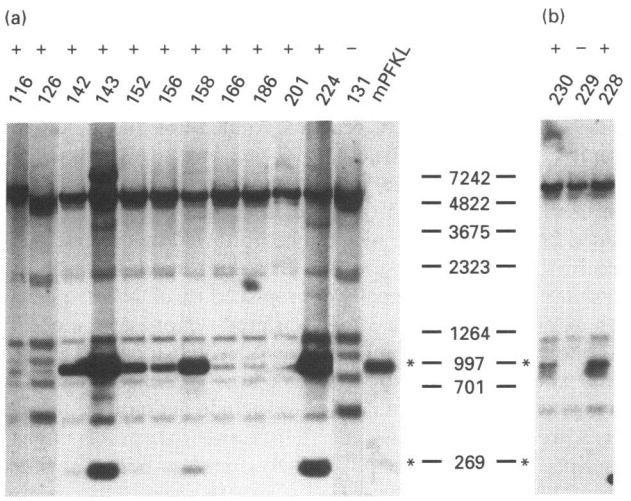

Figure 2 Southern-blot analysis of genomic DNA from selected mice born from microinjected oocytes

Lines propagated from individual mice bear the same numbers as the mice shown here. Blots were hybridized with the probe depicted in Figure 1; the two bands diagnostic of the transgene are marked with asterisks. mPFKL, *Pvu*II-digested PFKL mini-gene, identifying the diagnostic bands; (+) and (−) signs above serial numbers of the mice indicate transgenic or control mice respectively. (**a**) Mice born from oocytes injected with the mutated construct; (**b**) mice born from oocytes injected with the non-mutated construct. Size markers are in bp.

the founders were bred into lines in which the transgene was found to be passed on in a normal Mendelian pattern of inheritance, and copy numbers remained unchanged from one generation to the next. The appearance and litter sizes of all mice were normal.

Analysis of PFKL transgene expression in selected tissues of Tg-PFKL mice

Eight lines of Tg-PFKL mice were analysed for transgene expression by determining the total PFK catalytic activity *in vitro* in several tissues. These experiments were carried out under conditions in which all types of PFK were maximally active, and the results obtained represent the activity of both the endogenous and transgenic components of PFK activity, as shown in Table 1. Overall, high copy numbers were needed to obtain an increase in PFK specific activity; of the lines examined, only mice from the four lines with the highest copy numbers (142, ten copies; 158, ten copies; 143 and 224, several dozen copies each) showed increased activity levels when compared with control littermates. Lines 228 and 230, which were generated with the non-mutated construct, and lines 156 and 152, which were generated with the mutated one, did not express enhanced PFK activities in any of the tissues analysed. This fact, coupled with the similarity in copy numbers of lines 228, 152 and 156, indicated that the introduction of the mutation at the 3′ untranslated region was not the cause of the relatively low expression levels obtained per copy of transgene.

In the adult tissues sampled, PFKL is known to predominate in liver and erythrocytes, comprising 75% and 54% of all PFK subunits present respectively. PFKL is less prominent in adult brain (12%), heart (10%) and kidney, and is almost non-existent in skeletal muscle (Meienhofer et al., 1979; Dunaway et al., 1986, 1988, 1990; Dunaway and Kasten, 1988, 1989). Hence, correct tissue-specificity of transgene expression was expected to affect PFK activities mainly in the liver and erythrocytes. When PFK activities were measured in the livers of mice from the four lines with the highest copy numbers, from 132% (line 142) to 204% (line 143) of control littermate values were found (Table 1). The increase in activities were found to correlate roughly with the copy number of the individual lines. Erythrocyte PFK levels were found to be increased only in lines 158 and 143, to 147% and 130% of control levels respectively. No changes were found in the activity levels of PFK in samples obtained from transgenic brain, heart and, except for line 224, kidney. The results presented here were obtained by analysis of whole organs and did not address the possibility that the transgene may have been expressed at entirely different levels in small and non-representative parts of the tissues.

As shown in Table 2, PFK specific activity in brain and heart showed clear age-dependence, with the values obtained from younger animals, aged 6–10 weeks, some 70% higher than those obtained from older ones of 13–18 weeks of age. A similar inclination was observed in liver PFK, although it did not reach statistically significant levels; no such trend was observed in erythrocyte PFK. This phenomenon was observed in both controls and transgenic animals to similar extents. The ratio between PFK activity in transgenics and age-matched controls was independent of age, indicating that this phenomenon was not related to the presence or absence of the transgene. The data in Table 2 also highlight the large differences in PFK specific activity among different tissues.

Expression of the transgene was also demonstrated by analysis

Table 1 Ratios between the specific activity of PFK in tissues of Tg-PFKL mice and controls

Each value was obtained by comparing four to eight Tg-PFKL mice with the same number of non-transgenic control littermates. Values are means ± S.E.M. *Statistically significant ($P < 0.002$) values. For the erythrocyte value of line 224, $p = 0.097$. nd, not done.

		Ratio				
Line no.	Copy no.	Erythrocyte	Liver	Brain	Heart	Kidney
230	~ 2–5	1.09 ± 0.08	1.12 ± 0.07	1.00 ± 0.02	nd	nd
156	~ 5–10	1.00 ± 0.07	0.96 ± 0.08	1.03 ± 0.01	nd	nd
228	~ 5–10	1.07 ± 0.02	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.07	nd
152	~ 5–10	1.07 ± 0.07	1.00 ± 0.02	0.92 ± 0.04	1.03 ± 0.11	nd
142	~ 10	1.03 ± 0.05	1.32 ± 0.07*	1.01 ± 0.02	1.11 ± 0.11	nd
158	> 10	1.47 ± 0.05*	1.54 ± 0.04*	1.00 ± 0.04	1.09 ± 0.03	1.02 ± 0.02
143	⩾ 10	1.30 ± 0.06*	2.04 ± 0.19*	0.99 ± 0.07	1.13 ± 0.15	1.02 ± 0.04
224	⩾ 10	1.11 ± 0.03	1.84 ± 0.06*	1.14 ± 0.11	0.91 ± 0.14	1.21 ± 0.03*

Table 2 Specific activity of PFK in two age groups of transgenic and non-transgenic mice

Enzyme activity, expressed as units/mg of protein, in tissues of mice from two age groups were measured. Values represent means ± S.E.M.; identical age-dependencies were measured in Tg-PFKL and non-Tg littermates. The numbers of mice analysed were: Tg-PFKL, $n = 8$–12; non-Tg, $n = 10$–19. *Statistically significant by t-test analysis.

	Tg-PFKL mice			Non-Tg littermates		
Tissue	6–10 weeks	13–18 weeks	Ratio	6–10 weeks	13–18 weeks	Ratio
Erythrocytes	$(7.0 \pm 0.8) \times 10^{-3}$	$(7.7 \pm 0.16) \times 10^{-3}$	0.90 ($p = 0.098$)	$(4.8 \pm 0.7) \times 10^{-3}$	$(5.3 \pm 0.1) \times 10^{-3}$	0.91 ($p = 0.100$)
Liver	$(2.3 \pm 0.23) \times 10^{-2}$	$(1.7 \pm 0.06) \times 10^{-2}$	1.35 ($p = 0.095$)	$(1.5 \pm 0.1) \times 10^{-2}$	$(1.1 \pm 0.04) \times 10^{-2}$	1.4 ($p = 0.096$)
Brain	0.50 ± 0.01	0.30 ± 0.03	1.65* ($p = 0.001$)	0.33 ± 0.008	0.20 ± 0.03	1.67* ($p = 0.001$)
Heart	0.27 ± 0.02	0.15 ± 0.02	1.80* ($p = 0.002$)	0.18 ± 0.01	0.10 ± 0.02	1.71* ($p = 0.002$)

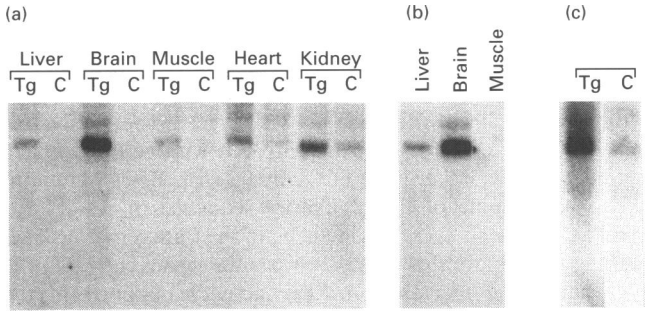

Figure 3 Northern-blot analyses of PFKL expression in total mRNA from tissues of PFKL transgenic mice

(**a**) RNA from separately pooled tissue samples of Tg-PFKL (Tg) and control (C) mice of line 158, probed with the oligomer probe which preferentially hybridizes with the transgene mRNA. (**b**) RNA from tissue samples of control mice probed with the 3′ untranslated probe which hybridizes only to PFKL mRNA. (**c**) Liver RNA from transgenic and control mice of line 143 probed with 3′ untranslated probe. Each lane contained 20 μg of total RNA. The integrity of the RNA and uniformity of the amounts loaded in the different lanes was checked by Methylene Blue staining (not shown).

of RNA from various tissues. The probe used in most of these analyses was a 35 bp-long oligonucleotide, the sequence of which was identical with that of the transgene in the region spanning the site of the 22 bp deletion; in the original undeleted sequence, there existed therefore a 22 bp-long mismatch which destabilized hybridization of the probe. Washing conditions in the Northern-blot analysis were empirically adjusted to favour retention of hybridization of the oligomer probe to the mutated transgene RNA. Under these conditions a signal was detected when transgene mRNA was present in a sample, whereas analysis of control RNA lacking the transgene yielded either a weak signal or no signal at all. In some cases the entire 3′ untranslated region of the mouse PFKL gene was used as a probe which hybridized specifically with the liver-type message, but was unable to differentiate between the transgene and endogenous PFKL mRNA. Using this probe, the presence of transgene mRNA was detected through an increase in the strength of the signal obtained when RNA samples from transgenic mice were compared with controls.

Analyses of RNA from samples of liver, brain, heart, skeletal muscle and kidney from lines 158, 143 and 224 showed that the transgene was expressed in all of these tissues to varying degrees. A representative Northern blot of RNA from tissues of mice from line 158 probed with the transgene-specific oligomer probe is shown in Figure 3(a). Transgene-specific bands were visible in all tissues, with the strongest one seen in brain RNA. No bands were visible in most of the control RNA lanes; those present in the lanes containing RNA from heart and kidney of control mice were probably due to residual hybridization of the oligomer probe with endogenous PFKL mRNA. These bands were much weaker in intensity than the corresponding bands present in RNA from similar organs of transgenic littermates, attesting to the expression of the transgene in these organs as well. Hybridization of similar blots prepared from tissues of the non-expressing lines 156 and 230 with the oligomer probe did not yield any signals (not shown), indicating that the transgene was not transcribed in these lines. In lines 143 and 224, transgene expression levels in liver were strong enough to permit detection of a difference in the amounts of total PFKL mRNA (endogenous compared with endogenous plus transgenic). This was demonstrated by hybridizing liver RNA from transgenic and control mice with the liver-specific 3′ untranslated probe and observing a difference in hybridization intensities, as demonstrated in Figure 3(c). Figure 3(b) demonstrates the high variability in the levels of endogenous PFKL expression among various tissues. Comparison of the levels of transgene expression in liver, brain and muscle (Figure 3a) with those of the endogenous PFKL gene (Figure 3b) showed that the relative intensity of transgene expression in these tissues usually parallelled the normal pattern.

Comparison of PFK activity data with the results of Northern-blot analyses showed that, in some tissues, transgene mRNA was present despite the absence of change in total PFK activity. It should be noted that the two experimental approaches do not measure the same parameter and their results are not interchangeable. Measured PFK activity results from all three types of subunits, whereas the RNA analysis presented here was limited to the liver-type subunit, transgenic or endogenous. A lack of increased PFK activity, despite the presence of transgene mRNA, was found in brain, heart and kidney, where PFKL comprises a small part of the total complement of PFK subunits, but was not in liver where PFKL predominates. The normal pattern of PFKL expression can qualitatively account for this finding, as will be discussed.

Total PFK catalytic activity is increased in transgenic fetal brains and livers

PFKL comprises 12% of the subunits in adult rat brain, a value only a quarter of its 48% share in rat fetal brain (Dunaway and Kasten, 1988). In liver, the adult share of PFKL is 75% compared with 36% in embryos (Dunaway et al., 1990). As mRNA and activity analyses showed that the transgene was expressed at levels that did not alter PFK activity in adult brain, we believed

Table 3 PFK specific activity in brain and liver samples of Tg-PFKL and littermate control embryos at gestational days 14 and 17

Results are means ± S.E.M. for the number of determinations indicated in parentheses. *Statistically significant ($P < 0.025$) by t-test analysis.

Tissue	Line no.	Gestational day	10^{-2} × PFK specific activity (units/mg)		
			Tg-PFKL	Control	Tg/control
Brain	143	14	11.98 ± 0.19 ($n = 3$)	6.65 ± 0.11 ($n = 5$)	1.80*
	224	14	17.28 ± 1.14 ($n = 4$)	7.82 ± 0.81 ($n = 5$)	2.21*
Brain	143	17	13.42 ± 0.81 ($n = 15$)	7.38 ± 0.51 ($n = 8$)	1.82*
	224	17	12.22 ± 0.80 ($n = 9$)	6.00 ± 0.53 ($n = 6$)	2.04*
Liver	143	14	6.26 ± 1.08 ($n = 3$)	3.82 ± 0.16 ($n = 5$)	1.64*
	224	14	3.20 ± 0.45 ($n = 5$)	1.78 ± 0.15 ($n = 4$)	1.80*
Liver	143	17	8.70 ± 0.51 ($n = 10$)	4.08 ± 0.26 ($n = 6$)	2.13*
	224	17	6.97 ± 0.70 ($n = 9$)	3.75 ± 0.29 ($n = 6$)	1.86*

that it was possible to detect an increase in the specific activity of total PFK from embryo brain, where PFKL was normally relatively much more abundant. Heterozygote transgenic males from lines 143 and 224 were therefore mated with non-transgenic females. Pregnancies were terminated after 14 or 17 days of gestation, and total PFK activity in the brain and liver of each of the embryos was assayed. This was followed by identification of each embryo as transgenic or non-transgenic by Southern-blot or PCR analyses.

In both lines sampled, the specific activity of PFK from transgenic fetal brains was 180–221 % of control values (Table 3). Similar differences were recorded when brains from day 14 or day 17 embryos were analysed. Results of a similar nature were obtained when liver samples from transgenic and control embryos were analysed, with the PFK specific activity values of transgenics ranging from 164 to 213 % of those of controls. The detection of increases in PFK activity in brains and livers of Tg-PFKL embryos was in accord with the normal pattern of PFKL expression, although the absolute value of the increase in the case of embryonic liver was somewhat higher than expected, as will be discussed.

DISCUSSION

Mice transgenic for PFKL were constructed as a model system to enable the study of the effects of PFKL overexpression in DS, where the endogenous PFKL gene is one of many genes triplicated. In an attempt to reproduce the situation present in DS as faithfully as possible, the murine PFKL was used as the transgene and was placed under the control of murine PFKL regulatory sequences. In the course of preparing the PFKL minigene, the region spanning 2.9 kb upstream of the initiator ATG, where the putative murine PFKL promoter was thought to exist, was analysed. Comparison of the nucleotide sequence from the above region with sequences from the same region of the human PFKL gene revealed a striking similarity in the first 120 bp immediately upstream of the initiator ATG (D. Levanon and Y. Groner, unpublished work). This region contained four potential SP1 binding sites, followed by a cluster of transcriptional initiation sites located approximately 50–65 bp upstream of the initiator ATG (Levanon et al., 1989; Rongnoparut et al., 1991).

Analysis of transgene expression

Analysis of PFK activity in several tissues of mice from eight of the lines obtained showed that the presence of the transgene enhanced PFK activity by 30–100 %, mainly in the livers and erythrocytes in lines with high copy numbers. The rather low increases in PFK activity, when compared with copy numbers, may indicate that either most of the integrated copies were non-functional or some of the regulatory sequences necessary for expression of PFKL were not included in the transgene construct resulting in low levels of expression per transgene copy. On the other hand, the increases in PFK activity obtained in the expressing lines were similar to those observed in DS samples (Epstein, 1986; Anneren et al., 1987) and are therefore physiologically relevant for studying the disease.

Liver-type PFK comprises 75 % of all PFK subunits in human adult liver (Dunaway et al., 1990). The levels of PFKL activity of 184 % and 204 % compared with controls in lines 143 and 224 respectively and the detection of increases in total PFKL mRNA using the relatively insensitive 3′ untranslated probe, indicated that PFKL expression was more than doubled in these lines. These findings are consistent with the known relatively high expression of PFKL in adult liver. PFKL comprises 33 % of subunits in rat embryonic liver (Dunaway et al., 1990). An increase in PFK activity in transgenic embryonic liver was therefore expected and indeed found. However, the fact that the measured increase (to 200 % of control values) was similar to that found in adult liver, despite PFKL being relatively twice more abundant in the adult than in the embryonic organ, is not well understood at present, and suggests a control mechanism more sophisticated than simple gene dosage. A similar discrepancy has previously been described in erythrocytes from DS patients [summarized in Epstein (1986)]. The gene for the amyloid precursor protein maps to band 21q22 in humans, and to chromosome 16 in mice. Levels of one type of mRNA of this protein were found to be increased by about 500–800 % in DS fetal brain (Tanzi, 1990), whereas levels of the product in tissues of trisomy 16 mice were recently found to be much higher than expected on the basis of simple gene dosage alone (Holtzman et al., 1992). These examples suggest the possibility that the assumption that the extra copies of genes triplicated in DS are always expressed as their endogenous counterparts might not always be correct. It therefore seems that calculations of the change in PFK activity that are based on PFKL gene dosage can supply only a rough estimate of the change, not a precise mathematical prediction of it.

PFKL comprises only 12 % of PFK subunits in adult human brain (Dunaway and Kasten, 1988). Owing to the relative paucity of PFKL in adult brain (compared with PFKM plus PFKL levels), even a doubling of brain PFKL expression, as was

observed in liver, although detectable on a Northern blot using the transgene-specific oligomer, was expected to generate only small increases in brain PFK activity on the edge of the sensitivity of the activity assay used. This consideration is in line with detection of transgene mRNA in adult brain, which was not accompanied by an increase in enzyme activity *in vitro*. This line of reasoning led us also to predict that increases in PFK activity should be detected in brains of mice embryos, where the relative share of PFKL is fourfold higher (Dunaway and Kasten, 1988) This prediction was borne out when PFK activity levels of 184–191% of controls were measured in brains of transgenic embryos at gestational days 14 and 17.

Analysis of RNA obtained from heart and muscle samples from adult transgenic mice of the three lines of the highest copy numbers revealed that the transgene was expressed, to some extent, in these tissues; no enhanced PFK activity was noted in hearts from all eight lines examined. Again, this finding can be explained as in brain, as PFKL is almost non-existent in skeletal muscle and is rare in heart (Dunaway et al., 1986, 1988; Dunaway and Kasten, 1989).

Transgene expression is similar to that of the endogenous PFKL gene

Several findings indicated that the pattern of tissue-specificity with which the transgene and endogenous PFKL gene were expressed was similar in the tissues sampled. First and foremost among these was the finding that, in the tissues analysed, enhanced PFK activity was located in precisely the tissues where the normal expression levels of PFKL suggested it would be, and not in others. Transgene expression resulted in enhancement of PFK activity in liver and erythrocytes, the two organs where PFKL is most prominent. Expression of the transgene in adult brain and heart, at levels that did not result in any enhancement of PFK activity, was also in line with previous reports by other authors who showed that, although expressed, PFKL was relatively rare in these organs. The normal pattern of PFKL expression suggested that the transgene should be expressed at relatively high levels in embryonic brain and liver, as was indeed the case in the lines examined. This showed that testable predictions of transgene expression could be formed, and proven correct, on the basis of the known expression pattern of the endogenous PFKL gene.

Transgene expression in embryonic brain

Many of the symptoms of DS are already present in newborns (Tolksdorf and Wiedemann, 1981), indicating that the extra chromosomal material also exerts its influence during the fetal period of development. The finding that PFKL gene dosage affects PFK activity in embryonic, but not in adult, brain suggests that PFKL is overexpressed in brain at the time when this organ begins to be damaged in DS and opens the question of possible connections between the two phenomena, despite the relatively low expression levels of PFKL in adult brain which were previously thought to preclude such connections. From a broader point of view, this result also suggests that some gene-dosage effects might be temporally separated from some of their consequences. Temporal separation between cause and effect may further complicate determination of the connections between overexpression of specific genes in DS and their physiological results, underscoring the need for well-defined model systems for gene-dosage effects.

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REFERENCES

Anneren, K. G., Korenberg, J. R. and Epstein, C. J. (1987) Hum. Genet. **76**, 63–65
Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. E., Smith, J. A. and Struhl, K. (1990) Current Protocols in Molecular Biology, John Wiley and Sons, New York
Avraham, K. B., Schickler, M., Sapoznikov, D., Yarom, R. and Groner, Y. (1988) Cell **54**, 823–824
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Cox, D. R., Kawashima, H., Vora, S. and Epstein, C. (1984) Cytogenet. Cell. Genet. **37**, 441–442
Dunaway, G. A. (1983) Mol. Cell. Biochem. **52**, 75–91
Dunaway, G. A. and Kasten, T. P. (1988) Brain Res. **456**, 310–316
Dunaway, G. A. and Kasten, T. P. (1989) Mol. Cell. Biochem. **87**, 71–77
Dunaway, G. A., Kasten, T. P. and Kolm, P. (1986) J. Biol. Chem. **261**, 17170–17173
Dunaway, G. A., Kasten, T. P., Sebo, T. and Trapp, R. (1988) Biochem. J. **251**, 677–683
Dunaway, G. A., Kasten, T. P., Crabtree, S. and Mishkar, Y. (1990) Biochem. J. **266**, 823–827
Elson, A., Bernstein, Y., Degani, H., Levanon, D., Ben-Hur, H. and Groner, Y. (1992) Som. Cell Mol. Genet. **18**, 143–161
Epstein, C. J. (1986) The Consequences of Chromosome Imbalance – Principles, Mechanisms and Models, Cambridge University Press, Cambridge
Epstein, C. J. and Epstein, L. B. (1981) in Trisomy 21 (Down's Syndrome) Research Perspectives (de la Cruz, F. F. and Gerald, P. S., eds.), pp. 253–260, University Park Press, Baltimore
Epstein, C. J., Epstein, L. B., Cox, D. R. and Weil, J. (1981) in Trisomy 21, an International Symposium (Burgio, A. R., Fraccaro, M., Tiepolo, L. and Wolf, U., eds.), pp. 155–172, Springer-Verlag, Berlin
Epstein, C. J., Avraham, K. B., Lovett, M., Smith, S., Elroy-Stein, O., Rotman, G., Bry, C. and Groner, Y. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8044–8048
Gehnrich, S. C., Gekakis, N. and Sul, H.-S. (1988) J. Biol. Chem. **263**, 11755–11759
Groner, Y. and Elson, A. (1991) Encyclopedia of Human Biology, vol. 3, pp. 175–189, Academic Press, New York
Hogan, B., Costantini, F. and Lacy, E. (1986) in Manipulating the Mouse Embryo – A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY
Holtzman, D. M., Bayney, R. M., Li, Y., Khosrovi, H., Berger, C. N., Epstein, C. J. and Mobley, W. C. (1992) EMBO J. **11**, 619–627
Korenberg, J. R. (1990) in Molecular Genetics of Chromosome 21 and Down Syndrome (Patterson, D. and Epstein, C. J., eds.), pp. 105–115, Wiley–Liss, New York
Levanon, D., Danciger, E., Dafni, N., Bernstein, Y., Elson, A., Moens, W., Brandeis, M. and Groner, Y. (1989) DNA **8**, 733–743
Mattenheimer, H. (1970) in Micromethods for the Clinical and Biochemical Laboratory, Ann Arbor Science Publishers, Ann Arbor
Meienhofer, M. C., Lagrange, J. L., Cottreau, D., Lenoir, G., Dreyfus, J. C. and Kahn, A. (1979) Blood **54**, 389–400
Palmiter, R. D., Sandgren, E. P., Avarback, M. R., Allen, P. D. and Brinster, R. L. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 478–482
Rongnoparut, P., Verdon, C. P., Gehnrich, S. C. and Sul, H. S. (1991) J. Biol. Chem. **266**, 8086–8091
Summitt, R. L. (1981) in Trisomy 21 (Down syndrome) Research and Perspectives (De La Cruz, F. F. and Gerald, P. S., eds.), pp. 225–235, University Park Press, Baltimore
Tanzi, R. E. (1990) in Molecular Genetics of Chromosome 21 and Down Syndrome (Patterson, D. and Epstein, C. J., eds.), pp. 187–200, Wiley–Liss, New York
Tolksdorf, M. and Weidemann, H. R. (1981) in Trisomy 21, an International Symposium (Burgio, A. R., Fraccaro, M., Tiepolo, L. and Wolf, U., eds.), pp. 3–32, Springer-Verlag, Berlin
Uyeda, K. (1979) Adv. Enzymol. Relat. Areas Mol. Biol. **48**, 193–244
Van Keuren, M., Drabkin, H., Hart, I., Harker, D., Patterson, D. and Vora, S. (1986) Hum. Genet. **74**, 34–40
Vora, S. (1982) in Curr. Top. Biol. Med. Res., vol. **6**, pp. 119–167, Alan R. Liss, New York
Vora, S. and Francke, U. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 3738–3742

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