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Gene Dosage and Down's Syndrome: Metabolic and Enzymatic Changes in PC12 Cells Overexpressing Transfected Human Liver-Type Phosphofructokinase

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Abstract—*Down's syndrome (DS) is a human genetic disease caused by triplication of the distal third of chromosome 21 and overexpression of an unknown number of genes residing in it. The gene for the liver-type subunit of phosphofructokinase (PFKL), a key glycolytic enzyme, maps to this region and the product is overproduced in DS erythrocytes and fibroblasts. These facts, together with abnormalities which occur in DS glycolysis, make PFKL overexpression a candidate for causing some aspects of the DS phenotype. A cellular model for examining the consequences of PFKL overexpression in DS was constructed by transfecting rat PC12 cells with the human PFKL cDNA. Phosphofructokinase (PFK) isolated from PFKL-overexpressing clones was more inhibited by ATP and citrate and less activated by fructose-6-phosphate than control PFK; similar results were obtained when PFK preparations from DS and control fibroblasts were compared. In vivo NMR measurements determined that cells overexpressing PFKL performed glycolysis 40% faster than controls. These results show that overexpression of PFKL is the cause for altered biochemical regulatory characteristics of PFK in DS fibroblasts and can result in enhancement of glycolysis rates. It is also shown that increased gene dosage can exert its influence not merely by enhancing the amounts of gene products but also by altering their biochemical nature.*

INTRODUCTION

Down's syndrome (DS) is one of the most common human genetic diseases and occurs approximately once in 1000 live births (1). The disease is characterized by mental retardation, morphogenetic and biochemical abnormalities, and pathological manifestations resembling Alzheimer's disease that become apparent during the fourth decade of life (reviewed in 2, 3). Down's syndrome is caused by the triplication of the distal third of the long arm of chromosome 21, cytologically known as band 21q22 (reviewed in 4, 5). This region comprises approximately 0.5% of the human genome; it is estimated to contain between several dozen to a few hundred genes, of which only a fraction have been identified (summarized by 6). The precise mechanisms by which the disease is caused are unknown, but it is thought that the genes present in the excess genomic material are expressed in the same manner as their counterparts present in the normal DNA complement and that the resulting excess of normal gene products upsets the balance of biochemical reactions and other processes that ensure normal functioning of tissues and

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organs. It is believed that the DS phenotype is the result of the combined effects of overexpression of several genes or of groups of genes on chromosome 21 rather than overexpression of a single "DS gene."

Phosphofructokinase (PFK, EC 2.7.1.11) is a key glycolytic enzyme that catalyzes the transfer of a phosphate group from ATP to fructose-6-phosphate (F-6-P) to generate fructose-1,6-diphosphate (F-1,6-P_2). This reaction is considered to be a major rate-limiting step of glycolysis, and PFK is an extensively regulated enzyme that responds to a wide range of positive and negative effectors (reviewed in 7, 8). In vertebrates 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 (reviewed in 9). Each subunit is encoded by a separate gene; the one for the liver-type subunit of PFK (PFKL) maps to band 21q22.3, well within the "Down's region" of chromosome 21 (10–12), while PFKM and PFKP map to chromosomes 1 and 10, respectively (13, 13a). In various tissues the three subunit types randomly associate to form catalytically active homo- and heterotetramers, reflecting the relative proportions of each subunit type present (14, 15). Each organ exhibits a specific pattern of expression for each of the subunit genes, leading to a situation where the composition of the PFK tetramers is tissue-specific (16, reviewed in 9). The biochemical properties of PFK tetramers are dependent upon their precise subunit composition and vary among different tissues. This is interpreted to reflect the accommodation of the nature of the enzyme to the energy needs of each tissue (15, 17). Adaptation of this type is in line with evidence that the isoenzymatic profile and the biochemical properties of PFK change during development and aging (16–19) and in association with cancerous growth and progression (20–22).

The possible connections between DS and PFKL stem from the mapping of the PFKL gene to the "Down's region" of chromosome 21. Ample evidence has been published showing that the specific activity of PFK in erythrocytes obtained from DS patients is increased by 50% relative to control erythrocytes (summarized in 2) and a similar observation has been made more recently in fibroblasts (23). It has also been shown that the tetramer profile of erythrocyte PFK is abnormal in DS in that the relative amounts of PFKL-containing tetramers are increased at the expense of those that do not, probably as the result of the presence of added amounts of PFKL and of the random manner by which PFK subunits tetramerize (10). Yet, no information has been published on the possible effects of this change on the biochemical properties of PFK in DS. Some work has been published on glycolytic functions in DS. A decrease in F-6-P and an increase in F-1,6-P_2 in DS erythrocytes have been reported, both of which are consistent with enhanced PFK activity in vivo (24). The same authors also report a decrease in 2,3-diphosphoglycerate and an increase in ADP and AMP concentrations; ATP levels seem unchanged. When stimulated with methylene blue, DS erythrocytes performed glycolysis 20–40% faster than control cells. When labeled fructose is administered to DS infants, a smaller fraction of the labeled material is converted to glucose than in normal controls, and this has been interpreted as an indication of enhanced glycolysis in the DS patients (25). Abnormalities in the rate of cerebral glucose utilization in young DS adults have also been reported (26).

The abnormalities in DS glycolysis described above coupled with the chromosomal location of the PFKL gene and its overexpression in DS make PFKL overexpression a possible cause for some aspects of the DS phenotype. However, no direct proof of this has been presented; it could be argued that

the metabolic phenomena described above are the result of dosage effects of other yet-unidentified genes that are also triplicated in DS. Evidence of the role PFKL overexpression plays in DS can best be supplied by analysis of its effects in a well-defined system where PFKL is the only overexpressed gene. As part of our long-standing efforts to explain the molecular mechanisms by which overexpression of genes located in band 21q22 contribute to the symptoms of DS, we have developed cellular and animal models for studying gene dosage effects. The first gene investigated employing this approach was the one encoding copper–zinc superoxide dismutase (CuZnSOD), which also maps to the DS locus (reviewed in 27). It was found that cells overexpressing CuZnSOD had altered properties similar to those found in DS cells, such as diminished capabilities to accumulate neurotransmitters (28) and to release prostaglandins (29); transgenic-CuZnSOD mice exhibited several phenotypic features found in DS patients (29–32). To pursue these studies with additional candidate genes from the DS locus of chromosome 21, we have cloned and characterized both the cDNA (33) and gene (34) of PFKL. This work describes the construction of a cellular model system in which PFKL is overexpressed and the resulting influence on PFK regulation and cellular glycolytic rates.

MATERIALS AND METHODS

Materials. Aldolase, triose-phosphate isomerase, alpha-glycerophosphate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase as well as IEF agarose (type VIII) and IEF markers were purchased from Sigma. Cibachron F_3GA resin was purchased from Pierce Chemical Co. Lipofectin reagent was from BRL. Ampholines (Bio-Lyte 3-10) were purchased from Biorad. [^{13}C]glucose (99% pure) was from Omicron Biochemicals.

Construction of pSV-cPFKL Expression Vector. The pSV2-DHFR plasmid (35) was digested with HindIII and BglII, separating between the DHFR cDNA and the pSV2 backbone. The hPFKL cDNA was isolated from the plasmid cPFKL3.0 (33) by digestion with EcoRI and XbaI. The cDNA fragment contained 55 nucleotides of the 5′-untranslated region, the entire coding region, and ended in the 3′-untranslated region, one nucleotide upstream of the polyadenylation signal. The protruding ends of both the pSV2 backbone and the hPFKL cDNA were filled in and the fragments were purified by agarose gel electrophoresis and ligated together. The presence of a unique BamHI site 932 bp downstream of the initiator ATG enabled the identification of constructs with the proper orientation. Restriction enzyme digest of DNA, agarose gel electrophoresis, ligation, bacterial transformation, plasmid DNA preparation, and Southern and Northern analyses were as described in (36). Cytoplasmatic RNA was prepared by the RNAzol method of Chomczynski and Sacchi (37).

Cell Culture. PC12 cells were routinely grown in a 1:1 mixture of DMEM–F12 media, supplemented with 8% horse serum (HS), 8% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37°C and 5% CO_2. Primary human fibroblasts (strains KN6 and KDSC6) were prepared by finely mincing lung tissue obtained from a pair of age-matched DS and normal embryos electively aborted for therapeutic purposes. Cells were plated in DMEM supplemented with 20% FCS and glutamine, penicillin, and streptomycin as above and passaged until a population of healthy and homogenous morphology emerged. Fibroblast strains KN4 and KDSC4 were grown from abortus tissue at the genetics laboratory of the Kaplan Hospital, Rehovot. Strains 152 and 153, obtained from dizygotic twins discordant for DS (38), were a generous gift

of Dr. Charles Epstein, UCSF. All primary fibroblasts were routinely grown in DMEM supplemented with 20% FCS, glutamine, penicillin, and streptomycin as above. Population doubling time was determined by the MTT method (39).

Transfection of PC12 Cells with Lipofectin. Cells (1×10^6) were seeded in a 9-cm tissue-culture dish on the day prior to the transfection. The next day a total of 20 μg of DNA (10:1 molecular ratio between pSV-cPFKL and pSV-Neo) in 50 μl water were mixed with 50 μl of the Lipofectin reagent (BRL) in a polystyrene tube. The mixture was gently mixed and allowed to stand at room temperature for 15 min. Cells were washed twice with low-serum PC12 medium (0.5% FCS). Then 5 ml of low-serum medium were added to the plates, followed by gently adding the lipofectin–DNA mixture; the cells were then returned to the incubator. Twenty-four hours later, serum was added to a final concentration of 10% (1:1 ratio of FCS to HS), and after another 24 h the cells were split at a 1:10 ratio. Selection with G418 (Geneticin, Gibco, 350 μg/ml medium) was started 24 h later and stably transfected *neo*^{r} clones were isolated after three weeks.

Preparation of Genomic DNA from PC12 Clones for Southern Analysis and PCR. A 9-cm confluent plate was rinsed three times with Ca^{2+}/Mg^{2+}-free PBS. Cells were then lysed in 1 ml of NP-40 lysis buffer [10 mM Tris Cl (pH 7.6), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40], the lysate was spun at 10,000*g* for 10 min, and the nuclei-containing pellet was collected. Nuclei were then lysed in 300 μl of SDS lysis buffer [10 mM Tris Cl (pH 7.6), 10 mM EDTA, 10 mM NaCl, 0.5% SDS] to which 180 μg/ml of proteinase K were added and incubated overnight at 37°C. The DNA preparation was then gently extracted once with phenol–chloroform and three times with chloroform alone. Three volumes of isopropanol were added to the aqueous phase, and the precipitated DNA was spooled, air-dried, and dissolved in water.

PCR. Primers used for PCR were: TCCCCAGCCCCCACCCATGC (5′-primer) and GTGGATGCCCCAGGGCACTC (3′-primer). The 5′-most nucleotides of the primers were at positions 2368 and 2585, respectively, downstream of the initiator ATG (33), and specifically amplified a 218-bp-long human fragment. Reactions included 2 pmol of each primer, 2–5 μg of clone genomic DNA purified as described above, 70 mM Tris Cl (pH 8.8), 1 mM $MgCl_2$, 0.1% Triton X-100, 200 μM of each dNTP and 2 units of Taq polymerase. The amplification protocol consisted of 5 min denaturation at 94°C, followed by 35 cycles of 1 min denaturation, 1 min annealing (57°C) and 2 min elongation (72°C) and was terminated by a 10-min-long elongation step. Amplification was performed on a Programmable Thermal Controller (MJ Research, Inc.).

Partial Purification of PFK from Cultured Cells. The method used was based upon that of Dunaway and coworkers (40, 41). Cells ($50–100 \times 10^6$) were washed twice with PBS and resuspended in 4–6 ml of sonication buffer containing 50 mM Tris phosphate (pH 8.0), 25 mM NaF, 1 mM ATP, 10 mM DTT, 0.1 mM EDTA, 0.07 units/ml aprotinine, and 0.2 mg/ml PMSF. Cells were disrupted by sonication, and all subsequent work was performed at 4°C. The crude extract was spun at 10,000*g* for 15 min and the supernatant loaded directly onto a Cibachron F_3GA column previously equilibrated in column buffer [50 mM Tris phosphate (pH 8.0), 25 mM NaF, 0.1 mM EDTA, 0.05 mM F-1,6-P_2, and 10 mM DTT]. After loading, the column was rinsed with column buffer until absorbance at 280 nm returned to baseline values. Elution was performed with column buffer containing ATP and F-6-P (2 mM of each). Column fractions were assayed for PFK activity at pH 8.1 as described below; fractions in which activity was detected were pooled with the aid of Centricon-30 microcon-

centrators (Amicon) and stored at 4°C. PFK processed in this manner was purified by a factor of 20–30; levels of enzymatic activity remained constant for up to two weeks, during which the biochemical–kinetic assays described were performed. In contrast, PFK activity in crude extracts decayed rapidly with 50–80% of it lost after an overnight incubation at 4°C. Samples of PC12(−) and PC12(+) pools and of DS and control samples were always processed in parallel on the same day.

Partial Purification of Erythrocyte PFK. Blood samples used in this work were drawn in the course of routine periodic examinations of the donors. Samples were collected in heparin and stored at 4°C until processing (2–4 days). A total of 11 DS and seven control samples were used. DS and control samples were separately pooled and washed several times in equal volumes of 0.9% NaCl until no buffy-coat layer was detected. Cells were pelleted and lysed by resuspension in an equal volume of RBC hypotonic lysis buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.5 mM F-6-P, 1 mM EDTA, and 0.04% 2-mercaptoethanol), followed by gentle stirring in this buffer for an hour at 4°C. Cellular debris were pelleted by centrifugation at 140,000*g* for an hour, and the red supernatant was loaded on a Cibachron F_3GA column that had been equilibrated with column buffer as described above. The remainder of the purification protocol was identical to that described in the case of cultured cells. Hemoglobin did not bind to the column and was removed in the rinsing step prior to PFK elution.

Microassay of PFK Activity. PC12(+) and PC12(−) cells or DS and control fibroblasts were always processed and assayed in parallel. Cells ($4–7 \times 10^5$) were plated on 9-cm plates in regular growth medium. Three days later, while still not confluent, the plates were rinsed twice with Ca^{2+}/Mg^{2+}-free PBS and lysed by the addition of 1 ml of PFK lysis buffer (20 mM Tris Cl (pH 7.5), 10 mM NaF, 10 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 2 mM EDTA, 2 mM ϵ-aminocaproic acid, 10 mM DTT, 0.1 mM F-6-P, 0.07 units/ml aprotonin, 0.2 mg/ml PMSF, and 0.5% NP-40). The mixture was spun at 10,000*g*, 4°C for 10 min and the supernatant used for determination of PFK activity. No detectable PFK activity was found to be associated with the pellet. Assay of PFK activity was performed by coupling it to the disappearance of NADH, which was followed at 340 nm. Assays were performed in 96-well tissue-culture plates at room temperature (20–25°C). Equal volumes (5–15 μl) of the various samples to be assayed were placed in the different wells. After all the samples were dispensed, 300 μl of reaction mix were quickly added to each well, mixing effectively the reaction mix with the cellular supernatant and initiating the reaction in the process. (Reaction mix consisted of: 100 mM Tris Cl (pH 8.0), 10 mM KCl, 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM EDTA, 5 mM DTT, 0.4 mg/ml BSA, 0.14 mg/ml NADH, 2 mM F-6-P, 1 mM ATP, 1.5 units/ml aldolase, 5 units/ml triose-phosphate isomerase and 1 unit/ml alpha-glycerophosphate dehydrogenase.) The decrease in absorption of NADH was followed with a Titertek Twinreader (Flow Laboratories) at intervals of 1 min. Reaction rates reached maximal values 6–8 min after the initiation and persisted for another 6–8 min, during which the data were collected. Similar results were obtained when F-6-P was initially omitted from the reaction mix and after a preincubation step, the reaction was initiated by the addition of this reagent. One unit of PFK activity is defined as the enzymatic activity that converted 1 μmol of F-6-P to F-1,6-P_2 in 1 min under the conditions described. Total protein content of the supernatants was determined as described by Bradford (42) using bovine serum albumin as a standard.

Kinetic assays of PFK in the Presence of Effectors. Cellular or erythrocyte PFK purified as above was assayed in 96-well tissue-

culture plates. Preliminary assays at pH 8.0 were performed as described previously to determine the activity of PFK in the preparation used. The PFK preparations were then diluted with column elution buffer to similar activity levels such that 10 μl of the preparation could be used in each of the wells. Kinetic assays were performed at 37°C and at pH 7.35 in kinetic assay buffer (50 mM glycyl-glycine:KOH, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM $(NH_4)_2SO_4$, 0.5 mM EDTA, 5 mM DTT, and 1 mg/ml BSA) (21). Auxiliary enzymes and NADH were added as described above. ATP activation/inhibition assays were performed in the presence of 1.5 mM F-6-P and increasing concentrations of ATP. The reported concentrations of ATP and F-6-P in the different wells included the contributions of these compounds from the column elution buffer (0.05 mM each). F-6-P activation assays were performed in the presence of ATP concentrations that had resulted in maximal activation of PC12(−) or control fibroblast PFK in the ATP activation/inhibition assay. Citrate inhibition assays were performed in the presence of 1.5 mM F-6-P and at slightly inhibitory ATP concentrations. All assays were performed in 96-well plates so that PFK activity at all the effector concentrations chosen and for both PC12(+) or DS and control PFK could be assayed simultaneously.

IEF Agarose Gel Isozyme Profile Analysis. Horizontal 0.5-mm-thick slab gels were cast and run in an LKB 2117 multiphor apparatus. Gels were composed of 1.2% IEF agarose, 0.5% NP-40, and 40 μl/ml gel of ampholines, pH range 3–10 (Bio-lyte 3-10, Biorad). Then 5–10 × 10^6 cells were washed twice with Ca^{2+}/Mg^{2+} -free PBS and collected in 1 ml PBS. Cells were pelleted and lysed in 200 μl of PFK lysis buffer and spun at 10,000*g* for 10 min. An equal volume of the supernatant was mixed with 2× IEF buffer (1% NP-40, 40 μl/ml ampholines as above). After prefocusing for 425 volt-hours (v-h), 20 μl (1–2 μg total protein) of each sample were loaded on the gel with the aid of paper loading strips and electrophoresis was continued for another 2800 V-h. Total run time was 5 h; care was taken to keep the temperature of the gel low (4–8°C) by circulating cool water through the plate on which the gel rested. A pI calibration curve was generated by running six pI markers with isoelectric points in the pH range 7.2–5.1 in parallel to the PFK samples analyzed.

Staining for PFK Activity. Gels were stained in situ for PFK activity by pouring on the gel a solution composed of 100 mM Tris Cl, pH 8.1, 10 mM KCl, 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM EDTA, and 0.1 mg/ml BSA, to which the following were added: 3 units/ml aldolase, 12 units/ml triose phosphate isomerase, 10 units/ml glyceraldehyde phosphate dehydrogenase, 0.67 mg/ml NAD, 0.4 mg/ml nitroblue tetrazolium, 0.02 mg/ml phenazine methosulfate, 0.5 mM ATP, and 2 mM F-6-P. After several hours of incubation, intense blue bands appeared at the sites of PFK activity. These bands were absent from similar gels stained with the same solution as above from which F-6-P had been omitted, attesting to the specificity of the bands.

Electroblotting and Immunological Detection of PFK from IEF Gels. The IEF agarose gel was separated from the plastic backing on which it had been run and placed in contact with a nitrocellulose membrane that had been prewetted in electroelution buffer. Both gel and membrane were then placed between two prewetted 3-MM paper sheets and placed in the electroelution chamber. Electroelution was performed in a buffer containing 200 mM glycine, 25 mM Tris, and 0.1% SDS, final pH 8.3. Electroelution was performed for 50 V-h in a field strength of 3 V/cm. After the transfer, the blot was processed as in Towbin et al. (43), using skim milk (1% fat) as a blocker for nonspecific protein adsorbance. The primary antibody used was mouse monoclonal raised against a beta-galactosidase–hPFKL fusion protein and was produced by Dr. O. Leitner

of the Biological Services unit of the Weizmann Institute. These antibodies were capable of recognizing both the endogenous rat and the human liver-type PFK, but did not cross-react with the muscle-type subunit. Secondary antibodies were ^{125}I-labeled goat anti-mouse Fab'. Location of the bands was determined by autoradiography on Kodak RP5 film with an intensifying screen.

Cell Culture for NMR Techniques. PC12 cells ($20–30 \times 10^6$) were mixed together with 4 ml (dry volume) of Nunc Biosilon microcarrier beads that had been prewetted in PBS. The mixture was divided among several bacteriological petri dishes and incubated in PC12 growth medium for three to four days. On the day of the experiment, the beads with the adherent cells were gently placed in a 10-mm NMR tube and connected to a perfusion system of the type described in (44). Briefly, the perfusion system continuously circulated medium through the bed of beads while equilibrating it against a gas mixture of $95\%O_2/5\%CO_2$. The total volume of medium in the system was 50 ml and the circulation rate was 1.5 ml/min; the medium used was PC12 growth medium as described above, except that it contained only 1 mg/ml glucose. The tube with the cells was maintained at 37°C throughout the NMR measurements, in effect mimicking the conditions present inside a cell-culture incubator. Experiments with PC12 cells (see below) and with other cell lines (45) indicated that the cells seemed healthy and continued to divide while attached to the beads and in the NMR spectrometer.

NMR Measurements. Measurements were performed on a Bruker AM-500 spectrometer using a quadronuclear probe, which enabled software-controlled alternate measurements of ^{31}P and ^{13}C spectra without perturbing the experimental setup. [^{31}P]NMR spectra were recorded at 202.5 MHz by applying 90° pulses with a repetition time of 10 sec and composite pulse proton decoupling. ^{13}C spectra were recorded at 125.7 MHz by applying 60° pulses with a repetition time of 6 sec and composite pulse proton decoupling. ^{31}P spectra were collected for a period of 2 h, after which the perfused medium was changed to similar medium containing 1 mg/ml glucose labeled with ^{13}C at position 1 (99% enriched). ^{13}C spectra were collected in groups of 300 scans (30 min) for a period of 6–8 additional hours. Areas of the signals were obtained by integration and scaled in reference to the concentration of glucose in the medium at the beginning of the experiment (5.6 mM). The intensity of signals in the ^{31}P spectra was measured with the aid of the GLINFIT program (Bruker). ^{31}P spectra of the pools measured after the determination of glycolysis rates were similar to those measured beforehand, except for a general increase in the intensity of the signals. These findings indicate that the cells remained healthy and continued to divide while in the NMR spectometer. In some experiments lactate was measured enzymatically using lactate assay kit 826-UV (Sigma).

RESULTS

Construction of PC12 Cell Clones Overexpressing hPFKL. The human PFKL cDNA (33) was inserted into the pSV2 expression vector (46) as described in Materials and Methods, generating the expression construct pSV2-cPFKL (Fig. 1). Expression of the PFKL cDNA was driven by the SV40 early promoter; G418 resistance was conferred on recipient cells by cotransfecting this vector along with the pSV2-Neo plasmid (47). Initial attempts at transfection of the rat PC12 cell line (48) using the calcium phosphate coprecipitation technique failed; transfection was accomplished only through the use of the Lipofectin reagent (49). Of several dozen G418^r clones chosen for analysis, seven were found to have integrated pSV-cPFKL by Southern analysis of clone genomic DNA and were used in this study.

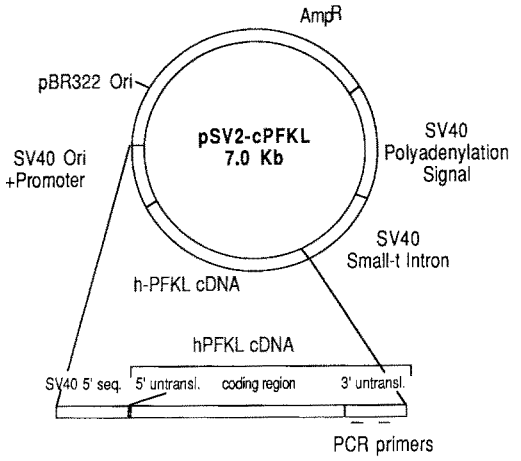

Fig. 1. Structure of the pSV2-cPFKL plasmid used for transfection experiments. Expanded region shows the hPFKL cDNA, including the approximate locations of the primers used for PCR amplifications and of the 3′-untranslated human-PFKL specific probe.

The probe used in this analysis was a human PFKL-specific probe derived from the 3′-untranslated region of the hPFKL cDNA (Fig. 1) (33). Alternatively, potential positive clones were identified by amplifying genomic DNA samples using the PCR technique (50). The primers used for amplification originated in the 3′-untranslated region of the human PFKL cDNA (Fig. 1) and specifically amplified the hPFKL sequences (Fig. 2).

Expression of the integrated cDNA was examined by Northern analysis of total cytoplasmatic RNA (Fig. 3). Probing with the human PFKL-specific probe described above revealed a human-specific mRNA present in most of the clones that had integrated the pSV2-cPFKL construct and absent from all of those which had not. The size of this mRNA was somewhat larger than the 3 kb of the endogenous rat and human PFK mRNAs (Fig. 3) because it included vector-derived SV40 sequences. Other plasmid constructs in which the hPFKL cDNA was placed under the control of the cytomegalovirus promoter were also prepared. However, transfection of these constructs as well as transfection of the pSV2-cPFKL plasmid into mouse (L-929)

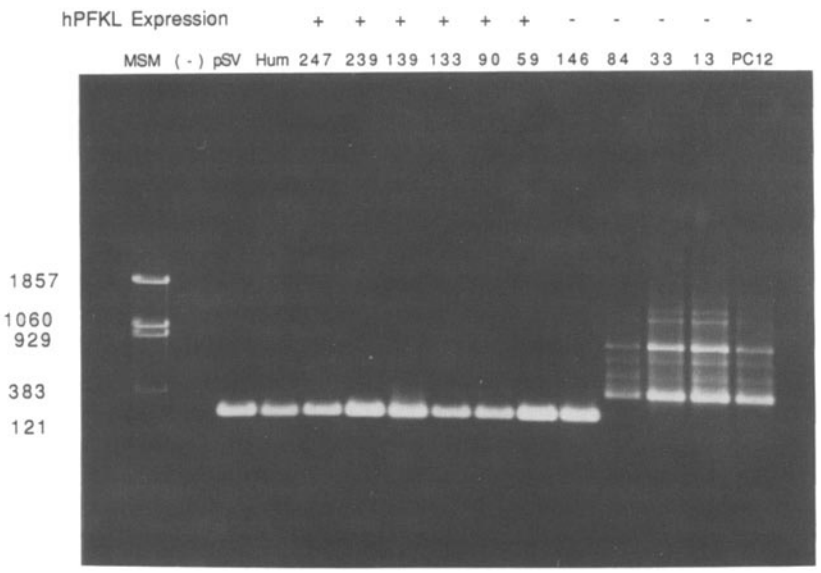

Fig. 2. PCR Detection of the pSV2-cPFKL DNA in stably transfected PC12 cells. Ethidium bromide-stained 1% agarose gel presenting the results of PCR amplification of selected PC12 clones using the primer pair described in Fig. 1. MSM, molecular size markers; −, control reaction to which no DNA was added prior to amplification; pSV, pSV2-cPFKL plasmid; Hum, human genomic DNA; PC12, untransfected PC12 cells; hPFKL expressor (+) or non-expressor (−) clones as judged by Northern and activity analyses are indicated above the clone numbers. Clone 146 has integrated pSV2-cPFKL but does not express it.

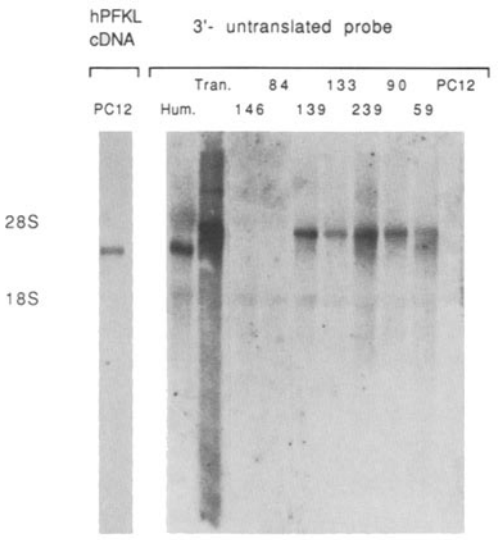

Fig. 3. Northern analysis of total cytoplasmatic RNA from PC12 clones. Right-hand panel was hybridized with the human PFKL-specific 3'-untranslated probe. Left-hand lane was probed with the entire hPFKL cDNA, which hybridized with endogenous PC12 PFK mRNAs. PC12, untransfected PC12 cells; Tran., RNA isolated from COS cells after transient transfection with pSV-cPFKL; Hum., human fibroblast (SV80) RNA.

and human (SV80) fibroblasts did not yield higher levels of PFKL expression (results not shown). Clones with integrated pSV2-cPFKL were analyzed for enhanced PFK enzymatic activity. The results, summarized in Table 1, showed that most of the clones that had integrated the hPFKL cDNA displayed a total PFK activity of 140–160% when compared to G418-resistant clones that were negative by DNA analysis. Perfect correlation existed between the presence of the hPFKL mRNA mentioned above and enhanced PFK activity. In general, the number of integrated copies of pSV-cPFKL in the clones was low (<3) as estimated from Southern blots (data not shown). Clone 59 was an exception to this rule and seemed to have integrated more than 10 copies. However, PFKL expression levels in this clone—as assayed by Northern and enzymatic activity analyses—were similar to those found in the other clones, suggesting that most of these copies were either partially or entirely not functional. Most of the subsequent work was performed on pools of negative [PC12(−)] and positive [PC12(+)] clones in order to obtain results based upon several independently derived transformants and thus avoid artifacts possibly present in specific clones. Pools containing two to four separate clones were mixed anew before performing major experiments to avoid overgrowth and domination of the pool by one of its constituents.

Introduction of the human PFKL subunit into rat cells raised the question of whether human–rat heterotetramers would form. Evidence supporting the existence of such heterotetramers was obtained from experiments in which the profile of PFK tetramers in the transfected cells was examined. Figure 4 depicts the result of isoelectric focusing (IEF) of crude protein extracts from positive and negative pools. When stained for activity (Fig. 4A), PFK from both pools displayed strong, diffuse activity localized in

Table 1. Relative Specific Activities of PFK in Sample PC12 Clones

PC12 clone	Average-fold increase in PFK specific activity[a]
PC12(−) clone	
13	0.94 ± 0.11 (8)
33	1.10 ± 0.10 (7)
77	0.95 ± 0.07 (4)
84	1.00 ± 0.08 (13)
146	1.01 ± 0.07 (8)
PC12(+) clones	
59	1.60 ± 0.19 (9)[b]
90	1.64 ± 0.16 (10)[b]
133	1.54 ± 0.27 (5)[b]
139	1.43 ± 0.19 (4)[b]
236	1.47 ± 0.15 (7)[b]
239	1.38 ± 0.09 (4)[b]
247	1.52 (1)[b]

[a]The specific activity of each clone in each assay was compared to those of negative clones assayed at the same time, giving rise to the fold increase value for each clone. The specific activity of PFK in clone 84 was 0.136 ± 0.080 units/mg protein and was used as the basis for comparison between the clones. Results are given as the mean and standard deviation with the number of repeats in brackets.

[b]$P < 0.001$ in comparison to clone 84.

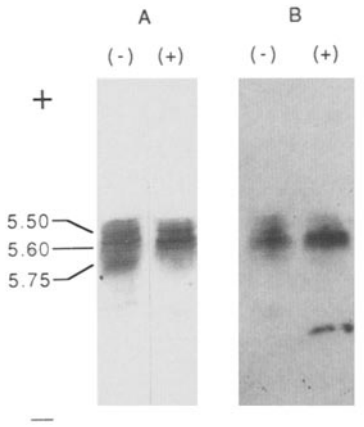

Fig. 4. Isoelectric focusing separation of PFK tetramer types on an agarose gel. (A) Panel stained for PFK activity; (B) panel blotted onto nitrocellulose and reacted with a monoclonal anti-PFKL antibody. −, PC12(−) pool; +, PC12 (+) pool. pI values of the bands were obtained from a calibration curve using pI standard markers as described in Materials and Methods.

the pI range of 5.75–5.4 in which several bands, representing the major tetramer species present, were visible. PC12(−) PFK displayed an additional area of activity centered around pI 5.75, somewhat removed from the bulk of the enzymatic activity, which was common to both pools. A Western blot prepared from a similar IEF gel was reacted with monoclonal antibodies raised against the liver-type subunit of PFK and that do not react with muscle-type PFK (O. Leitner, R. Zisling and A. Elson, unpublished results). Figure 4B shows that the tetramer composition of PFK in the two pools represented by the band pattern lit with the antibody was different, i.e., the intensity of the major band in the positive pool was stronger than that of the corresponding band from the negative pool; the two upper bands present in this latter pool were missing from the positive pool. Disappearance of bands from the PC12(−) lanes can most simply be interpreted as arising from the shift in tetramer composition resulting from the incorporation of the human L-type subunit into active PFK heterotetramers and reflects the relative depletion of tetramer types which do not contain the human subunit.

PFK Isolated from PC12(+) Clones or Down's Syndrome Fibroblasts has Altered Biochemical Properties. The altered isozyme profile of PFK in the PC12(+) pool raised the possibility that the enzyme's biochemical properties were also changed. We therefore examined whether PFK from pools of positive and negative clones responded differently to three known effectors of the enzyme, ATP, citrate, and fructose-6-phosphate (F-6-P). To this end, PFK was partly purified from crude extracts of PC12(−) and PC12(+) pools according to the method of Dunaway and Kasten (41), using affinity chromatography with Cibachron F3GA resin as described in the materials and methods section.

Figure 5 (A–C) and Table 2 display the influence of ATP, citrate, and F-6-P on the activity of PFK isolated from PC12 pools. Depending on its concentration, ATP is known to be either an activator or an inhibitor of PFK at physiological pH (7). While the activation profile, ranging from 0.05 to 0.3 mM ATP, was similar for both pools, the inhibitory effect of ATP was greater for PC12(+) PFK than for the enzyme isolated from PC12(−) (Fig. 5A). The same trend was seen for PFK inhibition by citrate (Fig. 5B). When F-6-P concentrations were increased, the activity of PFK from PC12(+) increased more slowly than that of PFK from the negative pool (Fig. 5C). These results are consistent with one another and suggest that the addition of the hPFKL subunit to the endogenous PC12 PFK causes the resulting heterotetramer mixture to be inherently less activatable in vitro.

The question of the relevance of these findings to DS was next addressed by repeating the experiments in fibroblasts and erythrocytes obtained from DS patients. Three pairs of DS vs. age-matched normal control primary fibroblasts (described in Table 3) were employed; blood samples were

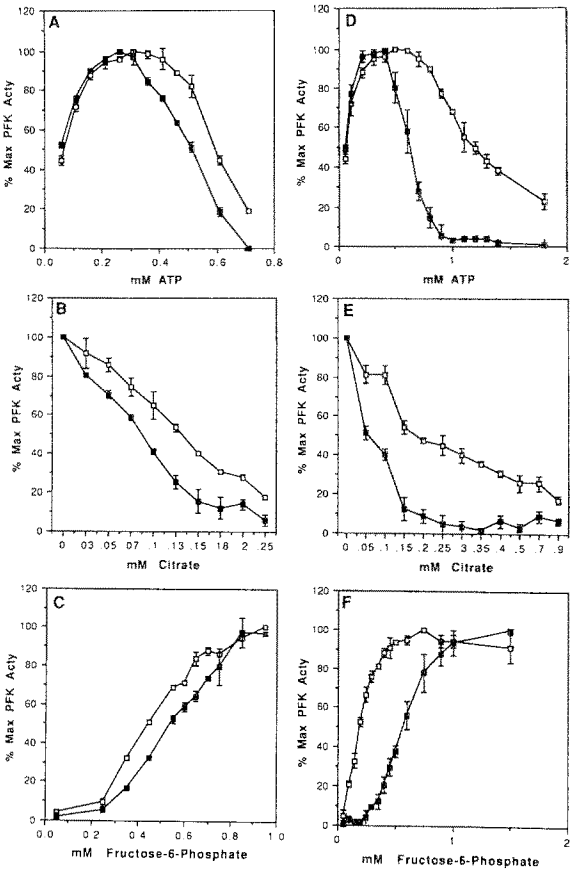

Fig. 5. Effects of ATP, citrate and fructose-6-phosphate on PFK activity. Partly purified PFK was assayed as described in Materials and Methods. Graphs A–C: PC12 PFK; graphs D–F: PFK from primary fibroblast strains 152 (control) and 153 (DS). Open squares, PC12(−) pool or strain 152; shaded squares, PC12(+) pool or strain 153. PFK activity at each point is compared to the maximal activity recorded in the assay at pH 7.35 and represents the average and standard deviation of two to four repeats. The remainder of the results are summarized in Table 2.

Table 2. Activation/Inhibition Parameters of PFK Isolated from PC12 Pools, Human Fibroblasts and Erythrocytes[a]

Cells	Ki, ATP (mM)	Ki, citrate (mM)	$S_{0.5}$, F-6-P (mM)
PC12(−)	0.62 ± 0.04	0.13 ± 0.01	0.44 ± 0.00
PC12(+)	0.50 ± 0.03	0.09 ± 0.00	0.57 ± 0.03
152	1.17 ± 0.07	0.18 ± 0.01	0.19 ± 0.01
153	0.62 ± 0.03	0.06 ± 0.01	0.57 ± 0.04
KN6	0.83 ± 0.01	0.11 ± 0.02	0.55 ± 0.05
KDSC6	0.71 ± 0.02	0.07 ± 0.01	0.70 ± 0.02
KN4	0.64 ± 0.03	0.18 ± 0.00	0.59 ± 0.13
KDSC4	0.50 ± 0.02	0.12 ± 0.01	0.75 ± 0.18
Control RBS	0.33 ± 0.02	0.40 ± 0.04	0.80 ± 0.03
DS RBC	0.31 ± 0.04	0.34 ± 0.05	0.88 ± 0.09

[a]Values represent the average and standard deviation of two to four repeats of each experiment. Differences between PC12(−) and PC12(+) values and between control and DS values were shown to be statistically significant ($P < 0.01$) using paired t test analysis on graphs of the type shown in Fig. 5. Differences between control and DS erythrocyte PFK were statistically insignificant.

Table 3. Characteristics of Primary Fibroblast Strains[a]

Strain	Phenotype	Karyotype	Gestational age	Tissue	Sex
152	Control	(46,XY)	20 weeks	lung	M
153	DS	(47,XX+21)	20 weeks	lung	F
KN6	Control	(46,XX)	20 weeks	lung	F
KDSC6	DS	(47,XY+21)	20 weeks	lung	M
KN4	Control	(46,XY)	12 weeks	embryo	M
KDSC4	DS	(47,XY+21)	12 weeks	embryo	M

[a]Listed are the primary fibroblast strains used in this work. Strains 152 and 153 were obtained from dizygotic twins as described in Weil and Epstein (38). The origin and maintenance of the other strains are described in Materials and Methods.

obtained from DS patients and non-DS mentally retarded controls residing under identical conditions in a home for the mentally retarded. The small volume of blood obtained from each donor necessitated the separate pooling of all DS and all control samples prior to PFK purification. Figure 5 (D–F) shows the influence of the three effectors on PFK from one of the pairs of fibroblasts and the entire set of results is summarized in table 2. The results obtained with the other two fibroblast pairs were similar to those obtained with the PC12 pools. Analysis of the results showed that PFK from DS fibroblasts was more inhibited by ATP and citrate and less activated by F-6-P, similar to the results obtained with the PC12 clones. It is therefore conceivable that overexpression of the L-subunit in DS fibroblasts constituted the cause for this change in the enzymatic properties of DS fibroblast PFK. Erythrocyte PFK, on the other hand, seemed to possess identical biochemical properties, irrespective of whether it originated in DS or in control samples. We believe that these different enzymatic response patterns stem from the large differences in the subunit composition of PFK in the two tissues, as will be elaborated upon in the discussion.

Determination of Glycolytic Rate and Major Phosphate Metabolites in PC12-PFK Pools by Nuclear Magnetic Resonance (NMR). The reaction catalyzed by PFK is considered to be a major point of regulation in glycolysis. It was therefore of interest to determine if hPFKL overexpression generated a detectable change in the rate of glycolysis and in the amounts of the major phosphate metabolites within the transfected PC12 cells. This question was addressed by the use of nuclear magnetic resonance (NMR), a noninvasive technique that enabled us to perform measurements on live, metabolically active cells (reviewed in 51). Techniques for growing cultured cells on microspheres compatible with NMR analyses have been described previously (44, 45) and are outlined in Materials and Methods. Cells inside the NMR spectrometer were continuously perfused with medium of the proper temperature and gas composition, in effect mimicking the conditions present in cell-culture incubators. Cells treated in this manner remained viable and continued to divide throughout the duration of the experiment (45 and A. Elson, this work).

Carbon-13 is the only carbon isotope detectable by NMR. Due to its very low natural abundance (1.11%), supplying cells with ^{13}C-labeled compounds makes it possible to follow the rates of processes in which these compounds are metabolized; nonlabeled compounds remain invisible and do not appear in the spectra collected. Pools of PC12(+) and PC12(−) were therefore perfused with medium containing [1-^{13}C]glucose. The rate of glycolysis in the cells was

followed by measuring the rate at which the labeled glucose was metabolized to lactate, most of which was secreted by the cells and accumulated in the perfusion medium. Consecutive [^{13}C]NMR scans were collected during successive periods of 30 min for a duration of 6–8 h; a sample of such spectra is plotted in Fig. 6. The consecutive spectra clearly show an increase in the intensity of the signal originating in C_3 of lactate with a parallel decrease in the intensity of the 1-β and 1-α glucose signals. Since the area of each peak is propotional to the concentration of the compound from which the signal is obtained, the spectra enabled us to estimate the rate at which the concentrations of glucose and lactate changed. These parameters were also influenced by the number of cells present in the sample assayed. Therefore, at the end of each experiment the cells were removed from the microspheres and counted; the data shown in Fig. 7 and listed in Table 4 are presented on a per-cell basis.

In general, the rate at which each cell carried out glycolysis varied slightly from one experiment to the next (Table 4) but was independent of the cell density on the microspheres at the cell densities used (results not shown). The doubling time of the positive and negative pools was found to be the same within experimental error and was much larger than the duration of the NMR measurements. Clone 90 was found to grow significantly slower than both pools (Table 4). However, due to the relatively short duration of the experiments, the increase in cell number in both pools and in clone 90 during the measurements was small and similar; it was therefore ignored in the calculations. When the rate of lactate accumulation in the medium was measured using enzymatic techniques it was found to be identical to the results obtained using NMR (data not shown).

As can be seen in Table 4, both the positive pool and clone 90 metabolized

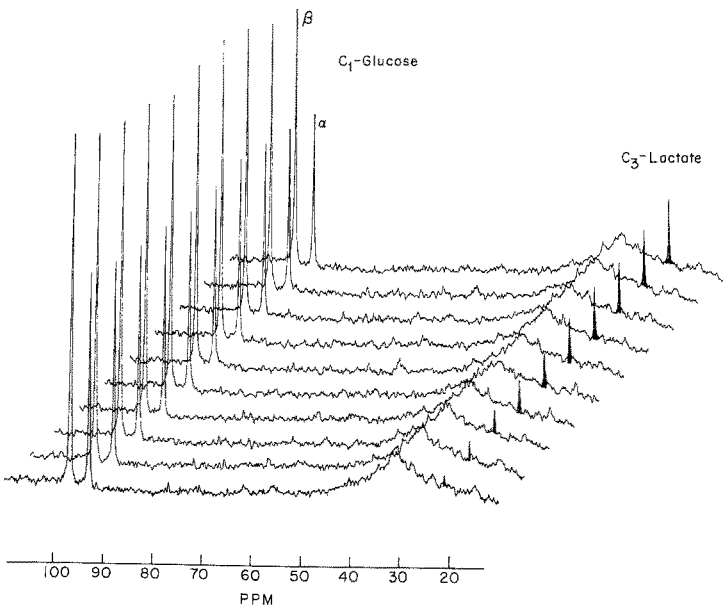

Fig. 6. Measurement of rate of lactate formation in PC12 clones by in vivo NMR. Drawn are consecutive ^{13}C spectra of the PC12(−) pool obtained in a representative experiment and which show the accumulation of lactate (shaded signal). The two peaks at 93 and 96.8 ppm originate in the alpha- and beta-enantiomers of glucose. Each trace represents the combined data collected in 300 scans measured over a period of 30 minutes, which is the time difference between consecutive traces. Spectra were drawn with a line broadening of 15 Hz.

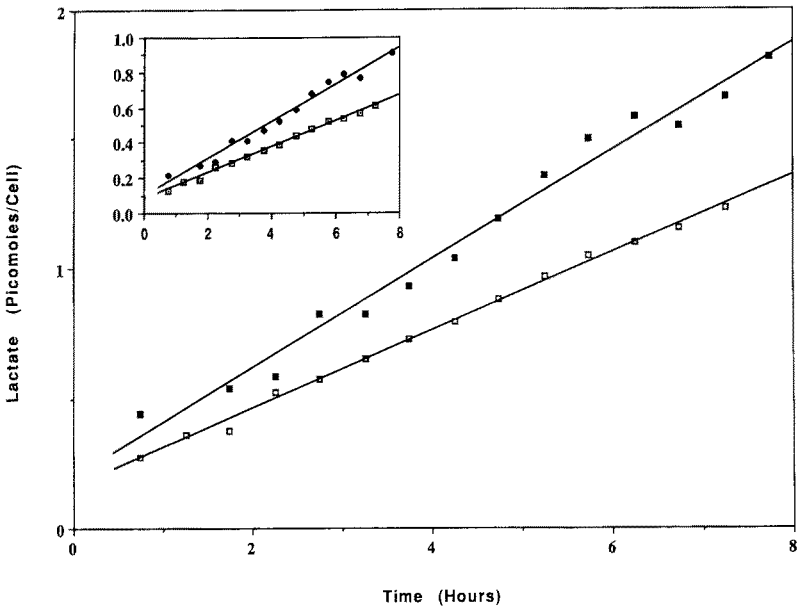

Fig. 7. Rate of lactate formation in vivo. Shown is the amount of lactate produced per cell as a function of time in PC12(−) (open squares) and in PC12(+) (shaded squares) pools as obtained in an experiment representative of those reported in Table 4A. The slope, representing the rate of lactate formation, of the PC12(+) pool is 40% larger than that of the PC12(−) pool. Inset: similar graph depicting calculated rate of glucose consumption.

glucose to lactate at a rate 39% and 21% faster than the control pools, respectively. Thus, despite its slower population doubling time, which could indicate a reduction in the overall rate of metabolism, clone 90 still carried out glycolysis at an elevated rate.

The naturally abundant isotope of phosphorus, ^{31}P, was also used in the NMR

Table 4. Rate of Lactate Production and Population Doubling Times of PC12 Cells

A. Rate of lactate production[a]

Cell culture	Rate of lactate formation (fmole/cell/hr)	-Fold increase relative to PC12(−)
PC12(−) pool	164.60 ± 15.28 (2)	
PC12(+) pool	228.52 ± 19.70 (2)	1.39 ± 0.02
PC12(−) pool	195.16 ± 2.34 (2)	
Clone 90	236.92 ± 7.54 (2)	1.21 ± 0.05

B. Population doubling times.[b]

Cell culture	Population doubling time (hours)
PC12 (−) pool	39.5 ± 3.4 (11)
PC12 (+) pool	35.6 ± 3.6 (6)
Clone 90	47.5 ± 4.8 (10)

[a]In each experiment a pair of cultures was processed consecutively using the same experimental set-up and their rate of glycolysis was compared, as shown in the fold-increase column. Results are the mean value and deviation of two repeats for each pair. Paired t test analysis of all four experiments showed that the difference found in glycolytic rates between control and hPFKL expressing cells was statistically significant ($P = 0.007$).

[b]Results are the mean value and standard deviation, with the number of repeats in parentheses. Clone 90 values are statistically different ($P = 0.01$) from those of both PC12 pools.

measurements. ^{31}P spectra of the cells recorded the relative steady-state levels of the major soluble phosphate metabolites (Fig. 8). Compounds detected were assigned on the basis of their chemical shift to the nucleoside triphosphates (NTP, mostly ATP), phosphocreatine, and the lipid precursors phosphoethanolamine and phosphocholine (Fig. 8). Since ATP is the major contributor to the NTP resonances, the intensity of these signals could be used to estimate ATP concentrations in both pools. Overall, no significant differences were found between the ^{31}P spectra of the PC12 pools, indicating that intracellular ATP concentrations were similar. This finding was further strengthened when the ratio between the intensities of the γ-ATP signals of both pools was found to be identical to the ratio of the cell numbers in both samples as determined by direct counting. On the other hand, no information regarding ADP concentrations could be derived from the spectra since the minor α and β ADP signals overlap with the dominant α and γ NTP signals, respectively. Protons are a product of PFK activity and of lactate production and the intracellular pH can be estimated from the pH-dependent position of the intracellular Pi signal. However, in the spectra of both pools the latter signal was obscured by the much stronger extracellular Pi signal, whose width is equivalent to approximately 0.2 pH units. We therefore conclude that the intracellular pHs of both pools did not differ from one another by more than this amount.

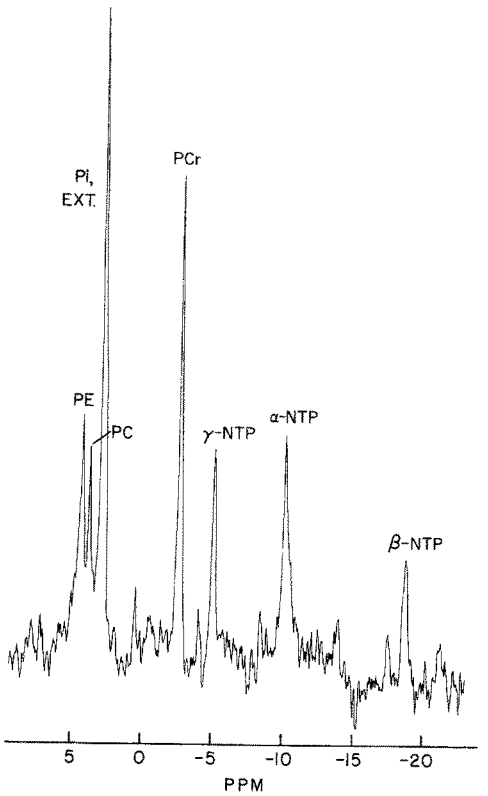

Fig. 8. ^{31}P spectrum of PC12(−) pool. The spectrum represents 720 scans taken over a period of 2 h and is drawn with a line broadening of 25 Hz. PE, phosphoethanolamine, PC, phosphocholine, Pi, EXT, external Pi; PCr, phosphocreatine, γ-, α-, and β-NTP, γ-, α-, and β-phosphoruses of the various nucleoside phosphates. PC12(+) spectrum was identical and is not shown.

DISCUSSION

We have described a cellular model system in which the human liver-type subunit of PFK is overexpressed, creating controlled conditions where some of the effects of PFKL overexpression can be dissected and their contribution to the DS phenotype evaluated. The model system consisted of rat PC12 cells in which the cDNA for the human liver-type PFK was expressed, resulting in a 50% increase in PFK activity in the cells. An increase of this magnitude is similar to what was found in DS erythrocytes (reviewed in 2) and fibroblasts (23), indicating that the data in this report did not result from levels of expression that are physiologically irrelevant. Attempts to achieve higher levels of PFK activity by placing the hPFKL cDNA under the control of the CMV promoter and using other fibroblast cell lines failed. Similar levels of increase in PFK specific activity have been obtained by transfecting mouse NIH 3T3 fibroblasts with a retroviral expression vector based on the human muscle-type cDNA (52). The reasons for the inability to achieve higher expression levels are unclear at present and may stem from the inherently

high levels of PFK in cultured cells (9) or from the use of cDNAs as opposed to genomic DNA segments in these vectors. Alternatively, it is possible that greatly enhanced PFK activity is detrimental to cells and the procedure of clone isolation effectively selects against high expressors.

We have shown that the hPFKL subunit formed catalytically active heterotetramers with the endogenous rat subunits, resulting in a change in the isoenzymatic profile of the cellular PFK. Similar findings have been reported for PFK from DS erythrocytes (10), where the added amounts of the L-subunit due to gene dosage distorted the distribution of the tetramer species and resulted in a bias towards those containing high proportions of PFKL. The formation of catalytically active human–rat heterotetramers showed that upon introduction into the rat cells, the human subunit behaved as the endogenous subunits did. This is in line with immunological and sequence-derived evidence, which suggests that the differences between the muscle- and liver-type subunits within a species are greater than differences between subunits of a similar type in different species (9, 33). We therefore believe that the results presented here stem from the added amounts of the liver-type subunit, rather than from having added a human subunit to the endogenous rat ones.

PFK isolated from PC12(+) pools displayed altered biochemical properties; it was more severely inhibited by ATP and citrate and exhibited reduced activation by F-6-P. Similar results were obtained when PFK preparations from pairs of primary fibroblasts obtained from DS patients and age-matched normal controls were analyzed. The correlation between the results obtained with PFK from the transfected PC12 cells and from the human primary fibroblasts suggests that the change in the biochemical properties of the enzyme in DS cells arises from the overproduction of the PFKL subunit and not, for instance, from overexpression of another unknown gene that modulates PFK activity. The results also show that relatively small increases in PFKL amounts both in the model system and in the DS cells were sufficient to cause this effect. A change in the biochemical properties of PFK could generate a situation where inappropriate levels of enzymatic activity would be produced in response to the normal metabolic signals that control PFK activity and glycolysis rates. Changes of this type are potentially harmful, especially when the strict control that exists on PFK subunit composition and biochemistry is recalled. On a broader scale, these results show that the commonly held belief that DS is caused by the added amounts of normal gene products should be modified to include cases, such as the one described here, where overexpression of a gene results also in an alteration of the biochemical properties of the active form of its product. We expect that effects of this type will be found in cases where the product of an overexpressed gene joins other polypeptides to form complexes whose subunit composition is not strictly defined.

Published work concerning changes of the PFK isozyme profile in different organs during development and malignancy suggests that rationalizing the direction of the effects induced by excess of the liver-type subunit on the biochemistry of the PFK isozymes is not straightforward, mainly because it is difficult to isolate the effects of adding PFKL from changes that occur simultaneously in the other subunit types. Dunaway and Kasten (17) showed that, during the development of heart and skeletal muscle, reduction in PFKL coupled with increase in PFKM amounts correlated well with reduced susceptibility to ATP inhibition and increased affinity to F-6-P, in good accord with the results presented here. The same authors also showed that the L_4 homotetramer was more severely inhibited by ATP and exhibited reduced affinity to F-6-P as compared to the M_4 homotetramer and that increases in

PFKL paralleled reductions in the total specific activity of PFK in rat brain (41). On the other hand, an increase in PFKL coupled to a decrease in PFKP resulted in a population of PFK tetramers that was less susceptible to ATP inhibition and displayed a higher affinity towards F-6-P, the opposite of what has been described here (16, 18). Similar conclusions regarding the nature of PFKL in tumors have been suggested by Vora et al. (22). It would therefore seem that the influence of adding more of one subunit type on the biochemical properties of the resulting PFK isozymes might not be the same in every case and would clearly depend upon the relative amounts of the three subunit types already present in the system.

In this light we interpret the fact that no significant differences were found in the biochemical nature of PFK obtained from DS erythrocytes when compared to controls. Erythrocyte PFK is dominated by PFKL (L/M/P subunit ratios of 56:40:4, respectively), while fibroblast PFK is dominated by PFKP (L/M/P subunit ratios of 22:17:61) (15). Accordingly, the biochemical nature of erythrocyte PFK is expected to be strongly influenced by the PFKL subunit prior to any changes; adding more PFKL might not elicit a detectable change in the biochemical properties of erythrocyte PFK, although it would result in more PFK activity being present. In fibroblasts, on the other hand, the predominance of PFKP should allow ample room for changes in the biochemical properties by adding PFKL, as indeed has been found. It has been suggested that even minor increases in the amounts of a given subunit might change the biochemical profile of the whole PFK complement (22, 53). This notion was based on the observation that the kinetic-regulatory properties of hybrid isozymes were unique and were not necessarily the average of those of the subunit types that made them up. This point could explain why a 50% increase in PFKL, which is not the major subunit type in fibroblasts, could change the overall biochemical properties of PFK in these cells.

Results of the NMR experiments showed that the increased specific activity of PFK measured in vitro under nonphysiological conditions (Table 1) was also manifested in vivo. This point is of some importance, since we have shown that PFK in the transfected PC12(+) cells was inherently less activatable, possibly in part compensating for the added amounts of enzyme. The NMR measurements showed that the end result of these conflicting changes was a net increase in PFK activity within the cells. The fact that the increase in the glycolytic rate of the positive pool ($\sim$40%) was similar to the increase in PFK specific activity (40–60%) suggested that under the physiological conditions within the cells, the enzymatic reaction catalyzed by PFK does indeed limit the rate of glycolysis. Information obtained from the ^{31}P spectra indicated that despite the measured increase in the rate of glycolysis, there were no significant changes in the profile of the major phosphate metabolites present in the cells. The finding that ATP concentrations were not changed despite the obviously increased rate of ATP production by glycolysis agreed with previously reported findings and indicated that the rate of ATP utilization in the cells was also increased (24).

In summary, we have demonstrated that in the transfected PC12 cells the added amounts of PFKL are incorporated into PFK tetramers, thereby changing the tetramer profile; the end result is more PFK of an altered biochemical nature. The metabolic consequences of these phenomena have been shown to include increased rates of cellular glycolysis and possibly ATP utilization. The significance of these findings with respect to DS stems from the fact that, with the exception of ATP utilization, similar changes have been shown to occur in the disease, thus forming a direct link between PFKL overexpression and certain abnormalities observed in DS patients. The results also

show that the relatively small (50%) increase in PFKL amounts in DS are sufficient to cause such changes. The harm PFKL overexpression can cause in DS is emphasized when it is placed in the context of the large body of evidence that suggests that the composition and biochemical properties of PFK are exquisitely fine-tuned to meet the changing metabolic requirements of the various tissues during development, aging and disease. Changes normally observed in the nature and amounts of PFK are thought to be responses to altered metabolic needs and are aimed at accommodating such needs. However, in the cases of DS and the model system described here, the changes in PFK are a direct consequence of the presence of an extra copy of the hPFKL gene and act to perturb the balance that tissues so decisively try to maintain, bringing about an incorrect metabolic state as we have shown here. The effects of adding an extra PFKL gene to a whole organism are expected to be more complex than can be examined in a cellular model. These effects should depend on the expression levels and relative amounts of each of the three subunit types and on the concentrations of the many effectors that are known to regulate PFK activity and hence be tissue-specific and developmentally regulated. Not all tissues are expected to be affected to the same extent or indeed even in the same direction; it is conceptually possible that subunit composition and concentrations of effectors in a given tissue will be such that adding PFKL will quite paradoxically reduce total PFK activity and, for example, slow down glycolysis. The construction of a transgenic mouse model for PFKL overexpression will serve to resolve at least some of the questions raised here and will shed more light on the contribution of PFKL overexpression to the DS phenotype.

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