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GENOMIC CLONES OF THE HUMAN LIVER-TYPE PHOSPHOFRUCTOKINASE

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Genomic clones of human liver phosphofructokinase (PFK) were isolated by screening a gene bank enriched for chromosome 21 sequences with two synthetic oligonucleotide probes designed from peptide sequences of purified human liver PFK. A 3.3 Kb fragment derived from the genomic clones was sub-cloned and designated pG-PFKL 3.3. It hybridized with a 3.5 Kb mRNA on Northern blots and was able to enrich selectively for liver PFK mRNA by hybrid-selection. These results demonstrated that the isolated clones contain sequences homologous to human PFKL mRNA. When hybridized to genomic DNA blots pG-PFKL 3.3 reacted with the same 3.3 Kb BamHI fragment in both human DNA and DNA of the mouse/human hybrid line WA17 which contains human chromosome 21 as the only human chromosome. These data confirm the assignment of the PFKL gene to chromosome 21.

Phosphofructokinase,(PFK, EC 7.7.1.11) which catalyzes the phosphorylation of fructose-6-phosphate to fructose 1,6 diphosphate, is an important mediator in the regulation of glycolysis (1,2). The mammalian enzyme is a tetrameric protein with a molecular weight of approximately 340,000 (for review see 3). In humans, PFK isozymes are encoded by three different loci: PFK-M (muscle), PFK-P (platelet) and PFK-L (liver) residing on chromosome 1, 10 and 21, respectively (4-7). The gene coding the liver subunit was more recently mapped to region q22 of chromosome 21 (8,9), a region known to be involved in Down's syndrome (reviewed in 10). As part of our long-term research effort to elucidate, at the molecular level, how an excess of the chromosomal segment 21q22 causes Down's syndrome,we wished to isolate the human gene encoding the liver PFK (PFK-L) and study its expression in Down's syndrome patients. The cDNA and gene of the rabbit PFK-M were previously cloned (11) and more recently, a cDNA of the human PFK-M was isolated (12). We report here the isolation and cloning of genomic fragments of the human PFK-L.

MATERIALS AND METHODS

To isolate chromosomal DNA containing human PFK-L sequence $6x10^4$ phages from a genomic library enriched for chromosome 21 sequences (13)(obtained from

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Millington-Ward and Pearson), were screened with end-labeled oligonucleotide probe (14). The SV80 cells are a continuous line of simian virus 40-transformed human fibroblasts (16). The mouse-human hybrid cell line WA17 was obtained from F.H. Ruddle. It contains three copies of chromosome 21 as the only human chromosome (17). SK-Hep-1 is a human hepatoma cell line (18,19). Other procedures used in this work have been previously described: RNA extractions and blot hybridization (20,21), DNA-blot hybridization (15), hybrid-selection and in vitro translation (22).

RESULTS AND DISCUSSION

Isolation of human PFK-L genomic clones. Homogenous preparations of human PFK-L were digested with trypsin, a few of the tryptic peptides were sequenced and two were used to synthesize oligonucleotide probes (unpublished data). Approximately $6x10^4$ λEMBL-3 phage plaques of a genomic library enriched for chromosome 21 sequences (13) were screened and seven phages (λgPFKL-1 to λgPFKL-7) that hybridized with both probes were isolated (Fig. 1). The finding that those seven phages hybridized to synthetic oligonucleotides generated from two independent peptides - strongly suggested that their inserts correspond to the human PFK-L. The λgPFKL clones also hybridized under stringent conditions to nick-translated human DNA but not to mouse DNA, confirming their human origin. Restriction mapping and hybridization to the oligonucleotide probe have indicated that five of the phages, i.e., λgPFKL-2, 4, 5, 6 and 7 are

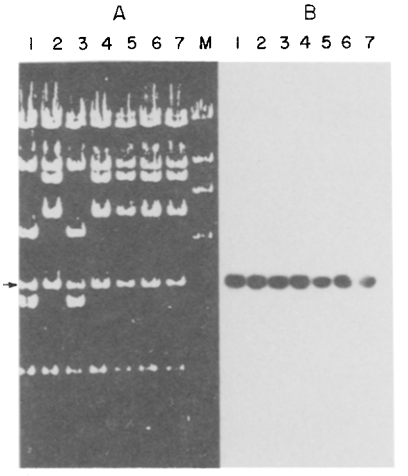

Figure 1. Analysis of λgPFK-L clones with the synthetic oligonucleotide probe. (A). Each of the isolated λgPFKL clones was digested with BamHI and Sal-1 and electrophoresed on 0.8% agarose gel. (B). Following transfer to Zeta-Probe blotting membrane it was hybridized with the end-labeled oligonucleotide probe as indicated in Materials and Methods.

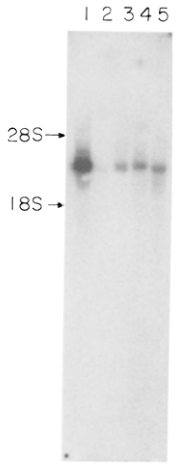

Figure 2. RNA transfer analysis. Poly(A)$^+$ RNA from: (1) SV80 cells (5 µg); (2) Mouse L-cells (2 µg); (3) WA17 cells (2.8 µg); (4) Human fetal liver (5 µg); (5) SK-Hep-1 cells (5 µg) was electrophoresed on 1.5% agarose-formaldeyhde gel as indicated in Materials and Methods. The RNA was transferred to nitrocellulose filter and hybridized with nick-translated pG-PFKL 3.3.

similar and share an overlapping region with the two other phages λgPFK-1 and 3, which are closely related to each other (Fig. 1). The 3.3 Kb BamHI fragment that hybridized with the oligonucleotide probe was sub-cloned in pGME-2 (designated pG-PFKL 3.3) and used to analyze RNA transfer blots.

Detection of PFK-L mRNA in human and mouse cells. Poly(A)$^+$ RNA was isolated from human fetal liver as well as from different cell cultures: SK-Hep-1 a human hepatoma line, SV80 human transformed fibroblasts, WA17 a mouse/human hybrid line containing three copies in human chromosome 21 and mouse L cells. When nick-translated pG-PFKL 3.3 was hybridized to the RNA blots a major band corresponding to an RNA of 3.5 Kb was observed in the human cultured cell lines, as well as in the fetal liver RNA (Fig. 2), and an additional minor RNA of ∿4.5 Kb was detected in SV80 cells (lane 1). A mouse RNA which seems to have a slightly smaller size also reacted with pG-PFKL 3.3 (lane 2), and accordingly in WA17 the RNA band was somewhat broader suggesting that both the human and mouse RNAs were included in this region (lane 3). Detection of the 3.5 Kb mRNA in the WA17 cells indicated that the human PFK-L gene is expressed in the hybrid line. Hybridization of the human PFK-L probe with mouse RNA of ∿3.4 Kb under stringent hybridization conditions, indicated a considerable degree of homology.

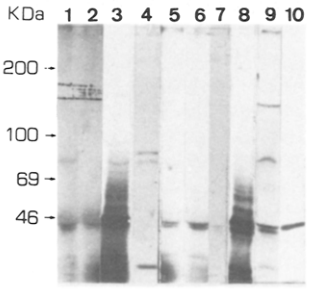

Figure 3. In vitro translation of hybrid-selected human PFKL mRNA. pG-PFKL 3.3 was linearized by digestion with SalI and used to hybrid-select PFKL mRNA from poly(A)$^+$ RNA of SV80 cells (22). The selected RNA was translated in vitro in the presence of [^{35}S] methionine (900 Ci/mmol) and polypeptides were resolved on a 7.5% SDS-polyacrylamide gel before and after immunoprecipitation with rabbit anti-rat PFKL serum; lanes (1 and 9) translation of RNA selected by hybridization to pG-PFKL 3.3 and immunoprecipitated with anti PFKL; lane (2) as in (1 and 9) but immunoprecipitated with non-immunized rabbit serum; lane (3) as in (1 and 9) but without immunoprecipitation; lane (4) in vivo labeled proteins extracted from SV80 cells immunoprecipitated with anti PFKL; lane (5) proteins translated by poly(A)$^+$ RNA from SV80 cells and immunoprecipitated with anti PFKL; lane (6) as in (5) but immunoprecipitated with non-immune serum; lane (7) as in 1 and 9 but RNA selected by hybridization to plasmid DNA containing the Ornithine decarboxylase cDNA (29); lane (8) as in (7) but without immunoprecipitation; lane (10) no RNA added.

Such an homology was also suggested by immunochemical studies (5,23). Indeed as presented below, rabbit anti-rat PFK-L serum (24)(kindly provided by G.A. Dunaway) efficiently recognized the human PFK-L. The liver isozyme is an ∿80K dalton (KDa) polypeptide (25,26). The amount of information required to code for such a protein corresponds to an mRNA of ∿2.5 Kb. We therefore assume that about 1 Kb of the PFK-L mRNA is untranslated.

Hybrid-selection and in vitro translation of the human PFK-L mRNA. The genomic subclone pG-PFKL 3.3 was used to select PFK-L mRNA. As shown in Fig. 2, poly(A)$^+$ RNA extracted from SV80 cells contains a relatively high proportion of PFK-L mRNA and was therefore chosen as the source for hybrid-selected RNA. The mRNA selected by hybridization with pG-PFKL 3.3 was then translated in a rabbit reticulocyte lysate and the products were electrophoresed on a SDS-polyacrylamide gel before or after immunoprecipitation with rabbit anti-rat PFK-L serum (Fig. 3). Even without immunoprecipitation a significant enrichment of an 80 KDa protein band was apparent in pG-PFKL 3.3 selected RNA (compare lane 3 to 8) and after immunoprecipitation it was the only protein band seen at that region (lanes 1 and 9). This band corresponded to one of the proteins which were immunopreci-

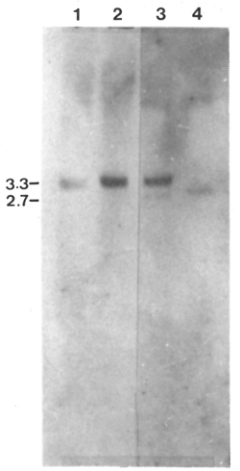

Figure 4. Detection of human PFKL sequences in human and mouse genomes. Ten micrograms of DNA was digestgd with restriction endonuclease, BamHI, electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose and hybridized to [^{32}P]labeled pG-PFKL 3.3. DNA was isolated from: lane (1) human blood cells; (2) human placenta; (3) WA17 cell line; (4) mouse L cells.

pitated from in vivo labeled SV80 cell extracts (lane 4). Since human PFK is a tetrameric protein (3) and fibroblasts contain relatively high proportions of the platelet PFK (27,28), we assume that the presence of two polypeptides, of ∿80 and ∿85 KDa, in lane 4 reflects the subunit composition of PFK in SV80 cells and that the upper 85 KDa band represents the platelet subunit co-immunoprecipitated with the liver one as PFK-(L+P) isozyme. The ∿80 KDa protein was absent in the pattern obtained without adding mRNA (lane 10), when the mRNA was not hybrid-selected (lanes 5 and 6) or when other recombinant plasmid DNA was used for hybrid-selection of mRNA (lanes 7 and 8). It was also absent when non-immune rabbit serum was used rather than anti PFK-L (lane 9). These results further support the conclusion that pG-PFKL 3.3 contains sequences homologous to the human PFK-L mRNA.

Detection of human PFK-L sequences in the mouse/hybrid line WA17. The genomic subclone pG-PFKL 3.3 was also used to assess the presence of human PFK-L sequences in the mouse/human hybrid line WA17. Human, mouse and WA17 DNAs were digested with BamHI followed by Southern blot analysis (Fig. 4). A 3.3 Kb fragment was detected in both human and WA17 DNAs, compatible with the BamHI

fragment in the λgPFKL clones (see Fig. 1). The mouse and WA17 DNAs contained a mouse homologous fragment of 2.7 Kb (Fig. 4). Digesting these DNAs with BglII and EcoRI gave similar results, i.e., the human fragments were present in the WA17 DNA (not shown). These hybridization results are consistent with the previous chromosome mapping data (obtained by immunochemistry and enzyme activity)(7,8,9)which assigned the PFK-L gene to the number 21 chromosome.

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