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Genetics

Mouse *sn*-glycerol-3-phosphate dehydrogenase: Molecular cloning and genetic mapping of a cDNA sequence

(brown fat/chromosome 15/restriction fragment polymorphism/untranslated RNA sequences)

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ABSTRACT **The isozymes of glycerol-3-phosphate dehydrogenase (GPDH; *sn*-glycerol-3-phosphate:NAD$^+$ 2-oxidoreductase, EC 1.1.1.8) in tissues of the mouse are coded for by two structural genes, *Gdc-1* and *Gdc-2*, located on chromosomes 15 and 9, respectively. In order to investigate the expression of these genes, we isolated a GPDH cDNA clone from a mRNA preparation isolated from brown adipose tissue. The GPDH cDNA clone was identified by colony hybridization and hybrid selection of a mRNA that was translated *in vitro* to produce immunoprecipitable GPDH protein. In blot analysis, the GPDH cDNA hybridized to a single mRNA species that migrated at the position of 23S ribosomal RNA. This GPDH cDNA clone was mapped to the *Gdc-1* locus by identification of a restriction enzyme polymorphism present in genomic DNA isolated from *Gdc-1* congeneic lines of mice.**

The isozymes of glycerol-3-phosphate dehydrogenase (GPDH; *sn*-glycerol-3-phosphate:NAD$^+$ 2-oxidoreductase, EC 1.1.1.8) in tissues of the mouse are coded for by two structural genes, *Gdc-1* and *Gdc-2* (1, 2), located on chromosomes 15 and 9, respectively. Biochemical and immunologic analyses of the isozymes derived from the expression of these genes indicate that protein structural homologies exist, although the amino acid sequences have not been determined (3). Because the *Gdc-2* locus is expressed at low levels, a comparative sequence analysis of the two loci will be most effectively made by determining the sequences of the genes.

Studies conducted by our laboratory and others on the GPDH isozymes have revealed intriguing developmental and genetic regulatory properties. First, the *Gdc-1* locus is almost exclusively expressed in solid normal and neoplastic tissues of the adult mouse, whereas *Gdc-2* is expressed in certain fetal tissues and ascites or cultured tumor cells (4). Second, the level of expression of *Gdc-1* in different tissues of the mouse, which varies over two orders of magnitude, is determined by a complex interaction of genetic and environmental factors (5). These factors have been partially characterized by the analysis of genetic variation extant in inbred strains and mutant stocks of mice (6, 7) and by environmental manipulation of established tissue culture lines (8, 9). Third, differences among species exist in the expression of GPDH isozymes (10–12) and in the regulation of expression by glucocorticoids (13).

To understand the nature of the genetic controls operating on the GPDH isozymic loci, it is important to determine the nucleotide sequence of the genes and to obtain molecular probes for quantifying mRNA levels. Toward the accomplishment of this objective, we have cloned a segment of GPDH cDNA from a mRNA population isolated from mouse brown adipose tissue, the tissue that has the highest known level of GPDH translatable mRNA (5).

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MATERIALS

Animals. C57BL/6J, BALB/cBy, and LT/Sv mice were obtained from the animal resources of The Jackson Laboratory. The C57BL/6J.Cas-*Gdc-1*^{d} congeneic line and *Mus castaneus* (cas) mice were obtained from Thomas Roderick, and the LT/Sv.Cas-*Gdc-1*^{d} congeneic mice were obtained from Eva Eicher and Janan Eppig. Mice were adapted to cold by housing them in a cold room at 4°C for 2 weeks.

RNA-Dependent Cell Free Synthesis of GPDH. Total RNA was obtained from tissues by the Labarca and Paigen (14) modification of the Cox (15) guanidine·HCl procedure. Poly(A)$^+$ RNA was isolated by chromatography on oligo(dT)-cellulose as described (16). Translations of RNA were carried out with the rabbit reticulocyte cell-free protein-synthesizing system (17).

Immunoprecipitation of products synthesized *in vitro* was carried out as described by Dobberstein *et al.* (18) with anti-GPDH antibodies prepared and characterized as described by Kozak and Burkart (3). Immunoprecipitated GPDH and total protein synthesized *in vitro* were further characterized on linear 3–18% gradient polyacrylamide gels with the buffer conditions described by Laemmli (19). Dried gels were fluorographed (20).

Purification of RNA on Agarose Gels. Twenty micrograms of poly(A)$^+$ RNA was electrophoresed in 1.25% Seaplaque (Marine Colloids, Rockland, ME) agarose gels containing methylmercury hydroxide as described by Bailey and Davidson (21). After electrophoresis the gel was stained with ethidium bromide and cut into 2-mm segments. Five milliliters (≈5 vol) of 0.01 M Tris·HCl, pH 7.4/1 mM EDTA/0.5% $NaDodSO_4$ was added to each segment of gel and heated at 70°C for 10 min. The solution was transferred to a water bath and incubated until the temperature reached 38°C. The poly(A)$^+$ RNA was recovered from the solution by chromatography on oligo(dT)-cellulose as described (16), except that the column was maintained at 38°C with a water jacket.

Blot Hybridization Gels. Detection of specific RNAs on agarose gels was performed as described by Derman *et al.* (22).

Molecular Cloning of Brown Adipose Tissue cDNA. Synthesis of double-stranded cDNA from C57BL/6J brown adipose tissue poly(A)$^+$ RNA was performed as described by Norgard *et al.* (23) for mouse liver RNA. Both the size and the yield of the S1-nuclease-treated double-stranded cDNA were similar to those described by Norgard *et al.* (23). From 48 μg of poly(A)$^+$ RNA [selected by two passes through oligo(dT)-cellulose], 9.9 μg of S1-nuclease-resistant cDNA was obtained.

Hybrid plasmid DNAs from both unfractionated and size-selected cDNA were used to transform calcium chloride-treated *Escherichia coli* strain MC 1061 (24). Transformants carrying inserts in the *Pst* I site of pBR322 were detected on agar plates

Abbreviations: GPDH, *sn*-glycerol-3-phosphate dehydrogenase; kb, kilobase(s).

by the absence of lactamase in the area surrounding a colony (25). In the several transformation reactions carried out, 40–80% of the transformed colonies had inserts in the *Pst* I site, and approximately 68,000 of these transformants were obtained per μg of cDNA.

Library Screening. Individual clones were grown in 96-well tissue culture cluster dishes. By using a replicate plating device with 48 1.5-mm-diameter stainless steel prongs, the clones were transferred onto nitrocellulose filters that had been placed on top of nutrient agar containing tetracycline at 25 μg/ml. After about 5 hr of incubation at 37°C, the filters were transferred onto nutrient agar containing chloramphenicol at 250 μg/ml. After overnight incubation, the colonies were prepared for hybridization by the procedure of Grunstein and Hogness (26).

When purified plasmid DNAs were used, 10 μg of DNA in 0.1 M NaOH was placed in a boiling water bath for 10 min, neutralized with $^1/_2$ vol of 3.82 molal Tris·HCl, boiled another 10 min, and quickly chilled in ice. A 7.5-μl aliquot of the final solution containing 1.0 μg of DNA was then dotted onto nitrocellulose filters as described by Thomas (27).

Southern Blots. Cellular DNA was isolated from mouse spleen nuclei. A nuclear pellet prepared from the spleens of two male mice as described by Berger *et al.* (28) was resuspended in 5 ml of 50 mM sodium acetate/10 mM EDTA, pH 5.1, and then adjusted to 0.5% $NaDodSO_4$. The DNA was extracted with phenol and precipitated with ethanol. The DNA was digested with restriction enzymes and 8.0 μg of DNA in each lane was electrophoresed in a 6.0-mm-thick slab gel of 0.8% agarose (29). DNA was blotted onto nitrocellulose paper as described by Southern (30).

Hybridization. Bacterial colonies grown on nitrocellulose, dot blots, and Southern blots were baked in a vacuum oven at 80°C for 2 hr and then hybridized either to a cDNA probe or to a nick-translated plasmid DNA probe. The cDNA probe was prepared from 1.0 μg of poly(A)$^+$ RNA as described (23). Plasmid DNA was nick-translated in the presence of [α-^{32}P]dCTP (31) to give a specific activity of at least 10^8 cpm/μg of DNA. Filters were pretreated for at least 5 hr in 0.45 M NaCl/0.045 M sodium citrate and 1× Denhardt's solution at 65°C. Hybridization to labeled probes occurred overnight at 70°C in 1× Denhardt's solution/0.9 M NaCl/0.09 M sodium citrate/0.5% $NaDodSO_4$/8.3 mM Tris·HCl, pH 8.0/10 μg of poly(adenylic acid) per ml/10 μg of poly(cytidylic acid) per ml/250 μg of yeast tRNA per ml/11.2 μg of denatured *E. coli* DNA per ml/62.5 μg of denatured salmon sperm DNA per ml (32). Filters were washed at 52°C in 15 mM NaCl/1.5 mM sodium citrate/0.05% $NaDodSO_4$ in a shaking water bath. After the filters were air dried, they were exposed to Kodak X-Omat film at −70°C with DuPont Lightning Plus intensifying screens.

Hybrid Selection. Ten micrograms of plasmid DNA was denatured as described above and then precipitated with 2 vol of ethanol. The DNA was resuspended in 10 μl of H_2O and placed in a boiling water bath for 10 min. The DNA was dot blotted onto nitrocellulose circles (27) that had been cut out with a no. 2 cork borer. Fifteen filters were pretreated at 50°C in 1.0 ml of 20 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.4/0.4 M NaCl/0.5% $NaDodSO_4$/65% (vol/vol) formamide. The filters were washed briefly in another 1.0 ml of hybridization solution at room temperature and seven or fewer filters were hybridized in 0.1 ml of hybridization solution containing 20 μg of brown fat poly(A)$^+$ RNA in a silane-treated 32 mm × 12.5 mm conical-bottom Nunc tube. The filters were hybridized for 3 hr at 50°C on a shaking platform. The filters were washed and the hybridized RNA was eluted as described (33). Ten micrograms of yeast tRNA was added as carrier, and the RNA was precipitated with 2 vol of ethanol and 0.2 M potassium acetate overnight at −20°C, dissolved in 100 μl of H_2O, and precipitated a second time at −70°C for 1 hr. The RNA pellet was washed with 70% (vol/vol) ethanol, dried, dissolved in 5 μl of H_2O, and translated.

RESULTS

We had previously estimated that approximately 0.7% of the translatable mRNA activity in the brown adipose tissue of cold-adapted animals was devoted to GPDH biosynthesis (5). Because *in vitro* translation data are biased toward lower molecular weight products (34), it is likely that the actual frequency could be as low as 0.3%. This is consistent with *in vivo* synthesis data (unpublished results). To maximize the probability of finding a GPDH cDNA clone, a cDNA plasmid library of 1,344 clones was prepared. By a combination of colony hybridization and dot blot analysis, one clone was chosen from the library and tested for its ability to select a translatable GPDH mRNA by hybridization.

The first screening of the clones involved detecting those colonies that gave an increased signal when hybridized with a cDNA probe made from cold-adapted poly(A)$^+$ RNA as compared to the signal obtained with the normal room temperature cDNA probe. Translation data shown in Fig. 1 indicate that about a 3-fold increase in translatable GPDH mRNA activity is present in cold-adapted brown fat. A comparison of the signals obtained with these probes is illustrated in Fig. 2. From this initial screen, 79 colonies, including colony C8 (denoted by arrow in Fig. 2), had greater hybridization with cold-adapted cDNA probes. These 79 colonies were screened by colony hybridization with cDNA probes made from poly(A)$^+$ RNA isolated from reticulocytes and neonatal brain (tissues low in GPDH activity) and with a sucrose gradient fraction enriched 5-fold for GPDH mRNA. Eight colonies that were negative with the reticulocyte and brain probes and positive with the sucrose gradient-purified probe were selected for further analysis by dot blots. Pu-

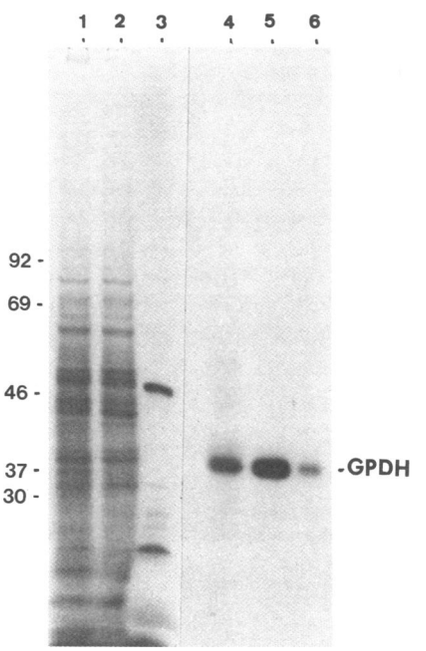

FIG. 1. Fluorogram illustrating the translation products synthesized *in vitro* by poly(A)$^+$ RNA isolated from mice kept at normal room temperature (lanes 2 and 4) and 4°C (lanes 1 and 5). Lane 3 shows the products synthesized with no exogenous RNA added. Lane 6 contains a purified [^{3}H]GPDH marker. Lanes 1–3 are the total translation products and lanes 4 and 5 are products precipitated with anti-GPDH antiserum.

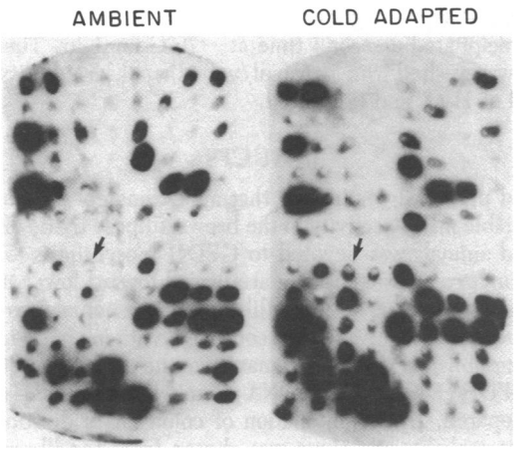

FIG. 2. Colony hybridization. Bacterial clones from the brown fat cDNA library were plated in duplicate on nitrocellulose filters. The filters were hybridized to cDNA probes made from poly(A)$^+$ RNA isolated from either ambient (normal room) temperature brown fat or cold-adapted brown fat. One set of duplicate filters is shown in the figure. Seventy-nine clones hybridized more strongly with the cold-adapted cDNA probe. The arrow indicates one of these clones, C8, which proved to be a GPDH clone.

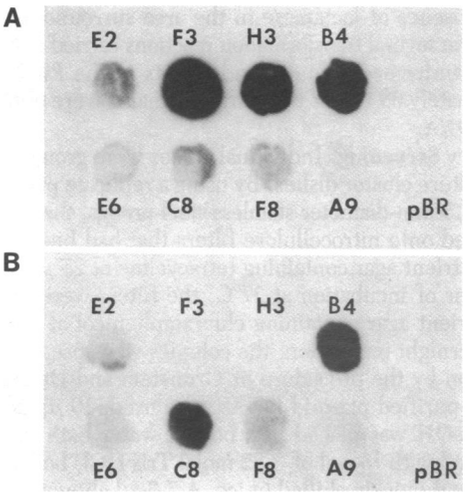

FIG. 4. Dot blots of eight cDNA clones and pBR322. Duplicate blots were prepared with purified plasmid DNAs. The blots were hybridized to a cDNA probe prepared from total cold-adapted brown fat poly(A)$^+$ RNA (*A*) or to a cDNA probe prepared from gel-purified fraction 2 poly(A)$^+$ RNA (*B*). C8 was the only DNA that hybridized more strongly to the fraction 2 cDNA probe.

rified plasmid DNAs from these eight clones were fixed to nitrocellulose filters and hybridized to cDNA probes made from total brown fat poly(A)$^+$ RNA and from a poly(A)$^+$ RNA fraction (fraction 2) obtained by agarose gel electrophoresis. GPDH is one of the major products detected when RNA in fraction 2 is translated (Fig. 3). Only one of the eight clones, C8, gave an increase in signal intensity when hybridized to the fraction 2 cDNA probe (Fig. 4).

The C8 plasmid was tested for its ability to hybrid select a GPDH mRNA. Plasmids B4 and F3 were also included as negative controls. Fig. 5, which shows a fluorogram of translation products immunoprecipitated with anti-GPDH antiserum and separated on NaDodSO$_4$/polyacrylamide gels, indicates that the C8 plasmid was capable of selectively hybridizing to GPDH mRNA (lane C8). The other plasmids gave a weak signal that was indistinguishable from two blank filter controls. Included also on the fluorogram are translation data which indicate that the RNAs recovered from two different hybridization solutions after removal of the filters were only 10–20% as capable of supporting protein synthesis as the starting RNA (compare lanes 4 and 5 to 6).

During purification of GPDH mRNA by agarose gel elec-

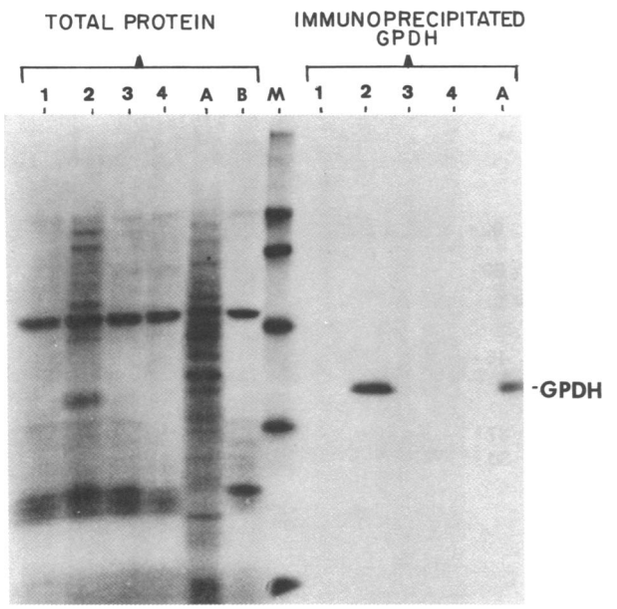

FIG. 3. A fluorogram of translation products synthesized *in vitro* by poly(A)$^+$ RNA. RNA was size fractionated on agarose gels. Lanes 1–4 contain translation products of RNA recovered from four consecutive 2-mm gel segments in the 23S region of the gel; lane A shows the products from the total poly(A)$^+$ RNA; lane B shows the control without RNA and lane M contains ^{14}C-labeled marker proteins (Amersham) with molecular weights of 14,000, 30,000, 46,000, 69,000, 92,000, and 200,000.

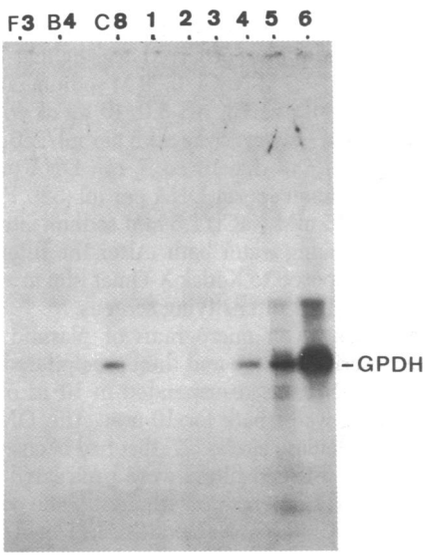

FIG. 5. Immunoprecipitable GPDH recovered after *in vitro* translation of RNA selected by plasmid DNAs. Lanes F3, B4, and C8 contain products of RNA selected by the respective plasmid DNAs analyzed by dot blot hybridization (Fig. 4). Lanes 4 and 5 contain the translation products of RNA recovered after the hybridization reaction and lane 6 contains GPDH synthesized by the untreated poly(A)$^+$ RNA. As negative controls we included blank filters (lanes 1 and 2) and a filter (lane 3) containing a chicken glyceraldehyde-3-phosphate dehydrogenase cDNA plasmid (41).

trophoresis in the presence of methylmercury hydroxide, translatable GPDH mRNA was detected only in the area of the 23S ribosomal RNA marker (Fig. 3). A blot with C8 DNA as a hybridization probe similarly detects hybridizable mRNA at the 23S position of the gel (Fig. 6). This corroborates the results of translation *in vitro*.

In order to obtain independent evidence that C8 contains sequences derived from the GPDH mRNA, we mapped the genomic location of sequences complementary to C8. If the C8 cDNA is specific for GPDH mRNA, then it should map to the *Gdc-1* locus on chromosome 15 because all detectable GPDH enzyme activity in the brown fat is coded for by *Gdc-1* (5). DNAs were prepared from mice carrying three different alleles of the *Gdc-1* locus. These alleles were previously shown to alter the heat stability or the electrophoretic migration of GPDH (35, 36). The DNAs were digested with *Bam*HI, *Eco*RI, and *Hin*dIII restriction endonuclease and then hybridized with nick-translated C8 DNA. Digestions with *Bam*HI resulted in a single 6.3-kilobase (kb) fragment with no apparent strain-dependent size polymorphism (Fig. 7*A*). *Hin*dIII digestion also produced one major fragment in each DNA. However, DNA from mice carrying the *Gdc-1*^{d} allele had a 5.9-kb restriction enzyme fragment (lanes 2, 3, and 4), whereas DNA from mice carrying the *b* or *c* alleles had a 7.9-kb fragment (lanes 1, 5, and 6). Because two of the strains of mice are congeneic at the *Gdc-1* locus for the *d* allele (lanes 2 and 4), the data indicate that C8 DNA is complementary to DNA on chromosome 15 in a region carrying the *Gdc-1* gene. In addition, a minor band of about 8.3 kb was present in lanes 2, 3, and 4. The origin of this band is currently unknown. However, the presence of an extra *Hin*dIII site within the *Gdc-1*^{d} allele is a possible explanation.

The *Eco*RI digestion showed no polymorphism among the different genotypes. However, the C8 probe detected two fragments, 1.60 and 0.76 kb, in all the DNAs (Fig. 7*B*). Because the minor 0.76-kb fragment in the *Eco*RI digests could be due

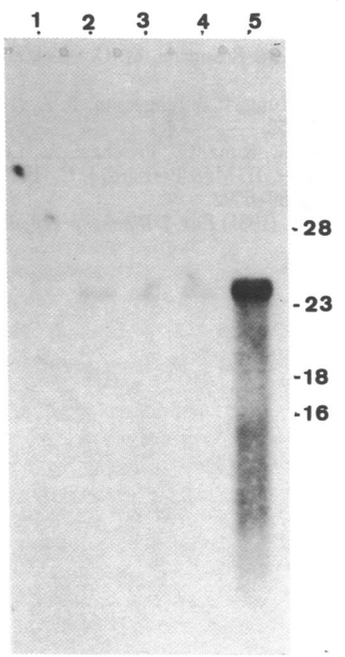

FIG. 6. Blot hybridization analysis of poly(A)$^+$ RNA isolated from lane 1, neonatal brain; lane 2, C57BL/6J cerebellum; lane 3, BALB/cBy cerebellum; lane 4, liver; and lane 5, cold-adapted brown adipose tissue. Blots were hybridized to nick-translated C8 plasmid DNA. Each lane contained 5 μg of RNA, except lane 1, which contained 10 μg. Numbers along the side refer to S values of ribosomal RNA markers.

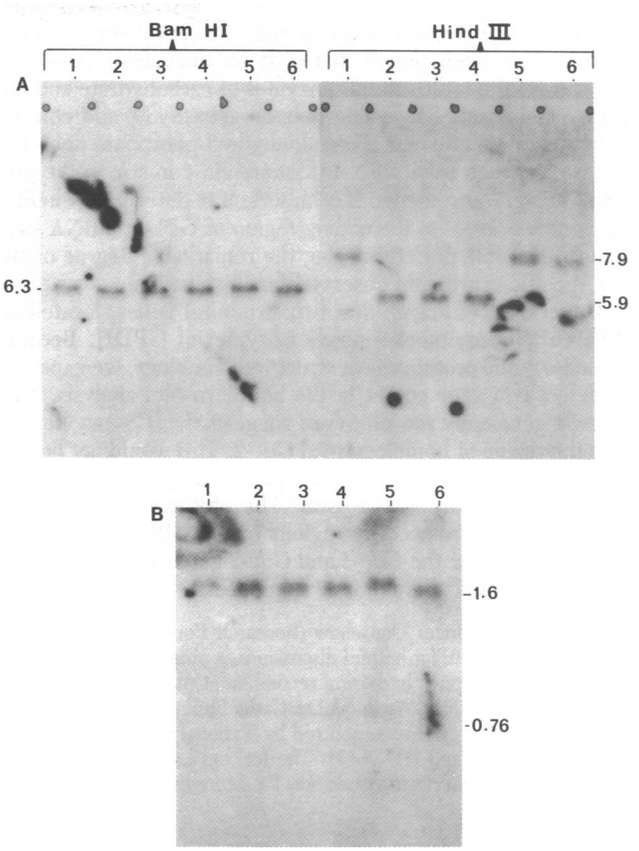

FIG. 7. Southern blot analysis of cellular DNAs hybridized to C8. (*A*) Cellular DNAs from B6-*Gdc-1*^{b} (lane 1), B6.Cas-*Gdc-1*^{d} (lane 2), Cas-*Gdc-1*^{d} (lane 3), LT.Cas-*Gdc-1*^{d} (lane 4), LT-*Gdc-1*^{b} (lane 5), and BALB/c-*Gdc-1*^{c} (lane 6) mice were digested with *Bam*HI and *Hin*dIII restriction enzymes. The numbers in the margin indicate the size of the DNA fragments in kb. (*B*) The same mouse DNAs were digested with *Eco*RI and the two fragments that hybridized to C8 are shown.

to a second gene or an *Eco*RI site within the region of *Gdc-1* homologous to C8 DNA, C8 DNA was digested with *Eco*RI and *Pst* I. The data (not shown) indicate that the C8 insert is approximately 350 base pairs long; it is excised with *Pst* I and it has an *Eco*RI site 60–80 base pairs from one end. Accordingly, the C8 probe has detected only one gene copy for GPDH.

DISCUSSION

Three independent lines of evidence support the conclusion that a GPDH cDNA sequence has been cloned. First, the C8 plasmid hybridizes to a mRNA that translates *in vitro* to produce a protein with the immunological properties and size expected for GPDH. Second, the blots of RNAs from three different tissues contained a single mRNA that gave a hybridization signal with the intensity expected for GPDH on the basis of enzymatic, immunological, and translatable mRNA data. Third, the C8 sequence maps genetically to a region of the mouse genome that carries the *Gdc-1* locus.

GPDH is composed of two identical subunits with a molecular weight of 37,000 (37). The amino acid compositions, the subunit sizes, and the peptide maps of mouse and rabbit GPDH are very similar (3). Because the rabbit amino acid sequence is known (38), the rabbit sequence data can be used to interpret data on the mouse gene. The rabbit GPDH subunit with 344 amino acids would require a coding sequence of 1,032 bases. However, the size of the GPDH mRNA is approximately 3,600 nucleotides. A mRNA this much larger than that required for coding is somewhat unusual, particularly when there is no in-

dication of mRNA size heterogeneity. Recently several other cDNAs have been cloned that code for enzymes of a similar type—i.e., the multisubunit enzymes of carbohydrate and energy metabolism. Rat lactate dehydrogenase (39) and chicken creatine kinase (40) and glyceraldehyde-3-phosphate dehydrogenase (41) each have mRNAs that are close in size to the protein coding requirements. It is important to determine whether sequences 5′ or 3′ to the coding region of GPDH mRNA have a functional role in determining the remarkable degree of tissue-specific expression observed for this enzyme.

Genetic data indicate that two structural genes, located on different chromosomes, encode isozymes of GPDH. Because these isozymic proteins have structural homology, we expected to detect two gene copies in the Southern blot analysis. That a single gene copy was observed suggests that C8 may be outside the region of homology with *Gdc-2*. This would not be surprising because C8 is only 350 base pairs in length and the mRNA has a large untranslated region. The characterization of the structure of the noncoding regions in the GPDH mRNA and the comparison of the *Gdc-1* and *Gdc-2* genes will require molecular cloning of the genomic sequences.

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