Isolation of partial cDNAs for rat liver and muscle glycogen phosphorylase isozymes

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Received 31 March 1986

cDNA clones for the rat liver and muscle glycogen phosphorylase isozymes have been isolated using isozyme-selective antibodies and libraries prepared in the expression vector, λgt11. A 1.2 kb cDNA coding for the carboxy-terminal domain of rat liver phosphorylase was found to have 82% homology with the amino acid sequence of rabbit muscle phosphorylase. Limited sequencing of rat muscle phosphorylase cDNA indicated a 95% homology with the rabbit muscle enzyme. The rat liver clone has eight additional amino acid residues at the COOH-terminus compared to the rat muscle clone. Furthermore, 17 of 26 (65%) residues between amino acids 815–840 differ between liver and muscle isozymes. The similarity in enzymatic properties and conservation of structure except at the COOH-terminus suggest that the liver and muscle phosphorylase isozymes do not exist in order to have significant differences in the regulation of glycogen breakdown in the two tissues. Rather, the phosphorylase isozymes probably evolved for tissue-specific transcriptional regulation of the genes in liver and muscle.

Phosphorylase Isozyme cDNA (Rat, Liver, Muscle)

1. INTRODUCTION

Glycogen phosphorylase (EC 2.4.1.1) catalyzes the first step in the intracellular degradation of glycogen. The amino acid sequence of the enzyme from rabbit skeletal muscle has been determined [1] and the crystal structure defined at 2.5 Å resolution [2,3]. Extensive investigation has defined the allosteric effectors and covalent phosphorylation involved in the acute regulation of phosphorylase (review [3–5]). Phosphorylase levels are regulated in both muscle and liver. For example, in developing muscle cultures phosphorylase activity increases following fusion of rat myoblasts into myotubes [6,7]. Phosphorylase activity also changes significantly between birth and weaning [8], in muscles of different fiber type [9–12], and with changes in muscle activity [13–15]. Total phosphorylase activity in liver is markedly depressed in the diabetic state [16–18], and evidence exists that triiodothyronine and insulin may be involved in regulating the expression of phosphorylase in hepatocyte primary cultures [19,20].

Phosphorylase exists as immunologically distinct isozyme forms in muscle and liver [21–23]. We report here the isolation of partial cDNA clones for both the rat liver and muscle isozyme forms of phosphorylase using isozyme-selective antibodies and λgt11 expression libraries. The cDNA clones allow analysis of phosphorylase mRNA expression in muscle and liver during the developmental and metabolic changes that have been previously defined only at the level of total enzyme activity.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2. MATERIALS AND METHODS

Glycogen phosphorylase was purified from rat liver and heart using modifications of published procedures [24]. Silver staining of SDS-acrylamide gels after electrophoresis of the purified enzymes from both tissues yielded a single band indicating the preparations were essentially homogeneous. Purified phosphorylase from the two rat tissues was independently used to immunize sheep. Initial immunizations were in complete Freund's adjuvant and boosts were performed in incomplete Freund's adjuvant at three week intervals. After the second boost the animals were bled one week later and serum tested for phosphorylase antibodies by Western blotting [25,26] and inhibition of enzyme activity. Positive serum was used for the affinity purification of phosphorylase antibodies. Purified liver or heart phosphorylase was coupled to CNBr-activated Sepharose (Pharmacia) using the manufacturer's instructions. The appropriate serum was repeatedly passed over the phosphorylase-Sepharose affinity column and washed extensively with phosphate-buffered saline. Adsorbed antibodies were eluted at pH 2.8 and immediately neutralized to pH 7.4 using 0.5 M sodium phosphate and then dialyzed extensively against 150 mM sodium phosphate, pH 7.4. Eluted antibodies were shown to recognize specific isozymes of phosphorylase by comparison of Western blots of crude homogenates of rat liver and heart and the purified enzymes as well as inhibition of phosphorylase activity. Purified isozyme-selective antibodies recognized a single protein of 92.5 kDa from the appropriate tissue from which the phosphorylase was purified. Isozyme cross-reactivity with the purified antibodies determined by Western blotting was 10% or less of the reactivity toward the isozyme form used for immunization. The isozyme-specific phosphorylase antibodies were further purified by passing through a column prepared by coupling a crude homogenate from *Escherichia coli* strain Y1090 to CNBr-activated Sepharose. The flow through containing the phosphorylase antibodies was used to screen λgt11 expression libraries [27] for the isolation of isozyme-specific phosphorylase cDNA.

Recombinant phages were adsorbed to *E. coli* strain Y1090 and plated at a density of 8–10000 plaque forming units per 15 cm LB-ampicillin plate with X-Gal and grown for approx. 4 h at 42°C as described by Young and Davis [27]. Nitrocellulose filters which had been previously soaked in IPTG followed by air drying were laid over the plates and β-galactosidase fusion protein synthesis was induced for 2 h at 37°C. Replica filters were washed and prepared for antibody blotting essentially by methods described previously [27,28]. Positive clones obtained in the first high-density screen were picked and taken through three successive rounds of antibody screening at low plaque density. The plaque purified positive clones were then used for large scale preparation of phage [27,28]. Isolated DNA was digested with *Eco*RI endonuclease and fractionated by agarose gel electrophoresis. Insert cDNA was subcloned into the M13Mp18 and M13Mp19 phage and sequenced using the dideoxy method of Sanger et al. [29].

The rat skeletal muscle cDNA library was constructed in λgt11 using the methods described by Huynh et al. [30]. Skeletal mulscle RNA was isolated by modification of the guanidinium isothiocyanate procedure of Chirgwin et al. [31]. Rats were killed by decapitation and skeletal muscle quickly removed for homogenization in guanidinium isothiocyanate. Following homogenization, sodium *N*-laurylsarcosine to a final concentration of 4% and 0.25 g CsCl/ml homogenate were added, and the samples layered onto 5 ml cushions of 5.7 M CsCl in 100 mM EDTA and centrifuged for 24 h in a SW28 rotor. RNA pellets were resuspended in SDS buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1% SDS) and heated briefly to 68°C. RNA was extracted with phenol/SDS buffer/chloroform (1:1:1), then chloroform alone (1:1) and then ethanol precipitated. Poly(A)$^+$ RNA was isolated by oligo-dT cellulose chromatography and used for preparation of cDNA. The rat liver cDNA library in λgt11 was a generous gift from Drs J. Schwarzbauer and R. Hynes [28].

3. RESULTS AND DISCUSSION

To isolate phosphorylase isozyme cDNAs, phage λgt11 expression libraries prepared using rat liver [28] or skeletal muscle RNA were screened using affinity-purified antibodies raised against

phosphorylase from the appropriate rat tissue. Fig.1 shows Western blots demonstrating that the antibody raised against liver phosphorylase has a strong selectivity for the liver isozyme, with a relatively weak cross-reactivity with the heart and skeletal muscle phosphorylase protein. The antibody raised against rat heart phosphorylase shows a similar selectivity, with a strong preference for the heart and skeletal muscle enzyme and weak cross-reactivity with the liver isozyme.

Initial plaque screening of approx. 3.2 and 1.6 × 10^5 independent liver and muscle clones, respectively, gave four putative liver clones and two putative muscle clones. The positive clones were purified and rescreened with the appropriate antibodies. Fig.2 shows a representative analysis of the fusion proteins from the purified clones. Lysogens from control λgt11 and clones containing positive immunoreactive proteins were prepared and induced with IPTG. Lysates were separated on SDS-acrylamide gels and subsequently analyzed by blotting with isozyme-selective antibodies. The major immunoreactive proteins with both the liver and muscle phosphorylase antibodies were the fusion proteins inducible by IPTG. Fig.2B shows a phosphorylase clone (λlp1) isolated from the rat

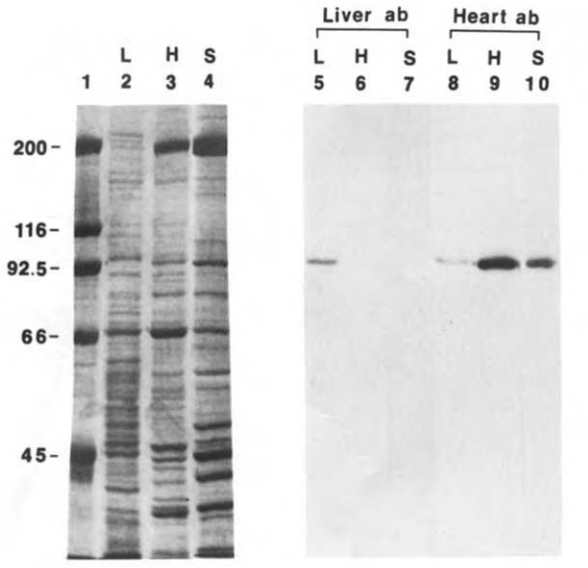

Fig.1. Isozyme-selective antibody blotting to rat liver, heart and skeletal muscle glycogen phosphorylase. 10000 × *g* supernatants of rat liver (L), heart (H) and skeletal muscle (S) were electrophoresed on 7.5% acrylamide-SDS gels [26]. Proteins were then electrophoretically transferred to nitrocellulose and incubated with purified anti-heart or liver phosphorylase antibodies. The filters were incubated first with rabbit-anti-sheep IgG, then in ^{125}I-protein A (10^5 cpm/ml). Both incubations were in a buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl (Tris buffer) and 0.25% BSA. Washes in Tris buffer with or without 0.05% NP-40 followed each incubation. Lanes: 1–4, Coomassie blue profiles of molecular mass standards and liver, heart and skeletal muscle extracts; 5–10, autoradiograms of Western blots developed using the isozyme-selective phosphorylase antibodies.

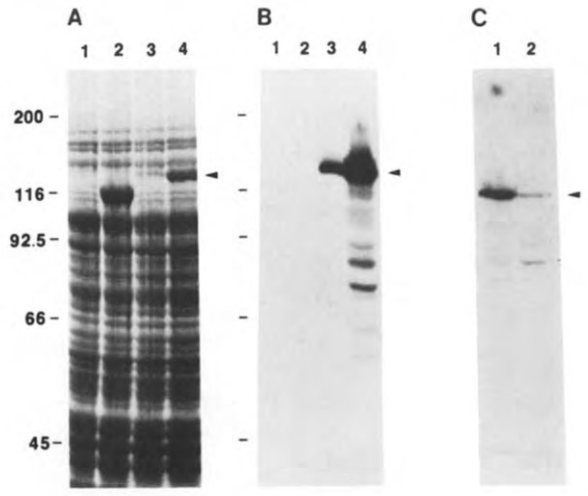

Fig.2. Characterization of phosphorylase cDNA clones by antibody blotting to β-galactosidase-phosphorylase fusion proteins. Bacterial lysates were prepared from the control lysogen Y1089/λgt11 (lanes 1 and 2) and lysogen Y1089/λlp1 (lanes 3 and 4) following inactivation of the repressor at 42°C and subsequent incubation in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of IPTG [27,28]. (A) The Coomassie blue staining profile of 7.5% acrylamide-SDS gels of the lysates. (B) The Western blot of a duplicate gel of (A) transferred to nitrocellulose and blotted with the liver-selective phosphorylase antibody. (C) Western blotting of liver and heart phosphorylase isozyme-selective antibodies to β-galactosidase-phosphorylase fusion protein. Equal amounts of the lysate containing the λlp1 β-galactosidase-phosphorylase fusion protein shown in panels A and B (lane 4) were electrophoresed on 7.5% acrylamide-SDS gels and transferred to nitrocellulose. Nitrocellulose strips were then blotted with purified antibodies raised against either liver (lane 1) or heart (lane 2) phosphorylase. The heart antibody binding to the λlp1 fusion protein was approx. 8–10-fold less than the liver antibody.

liver library and is representative of the inducibility of fusion proteins using IPTG following these protocols. The fusion protein of clone λlp1 (lanes 3 and 4) shows a strong immunoreactivity with the rat liver phosphorylase antibody. Importantly, clone λlp1 demonstrated the isozyme selectivity of the purified liver phosphorylase antibody. This is demonstrated in the Western blots shown in fig.2C using the liver- and muscle-selective antibodies and lysates from the IPTG-induced λlp1 clone. This finding indicated that a major isozyme antigenic domain was within the cDNA insert present in λlp1 which coded for a peptide of approx. 10 kDa. The insert in λlp1 was sequenced and the deduced amino acid sequence showed very strong homology with the carboxy-terminal domain of rabbit skeletal muscle phosphorylase confirming this as a phosphorylase cDNA (see figs 3 and 4).

The remaining liver clones and the two muscle clones were further analyzed by restriction mapping and DNA sequencing. The largest inserts identified were 1.2 kb (λlp2) and 1.1 kb (λmp1) for liver and muscle, respectively. Fig.3 shows the alignment of these two clones based on this analysis. All of the clones overlapped significantly coding for the carboxy-terminal region of phosphorylase, and contained at least one major isozyme antigenic domain as demonstrated by Western blotting of the induced fusion proteins.

The inserts of two liver clones λlp1 and λlp2 were entirely sequenced and two muscle clones λmp1 and λmp2 partially sequenced as outlined in

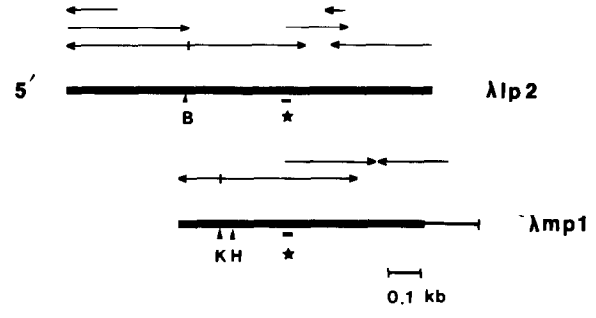

Fig.3. Sequencing strategy for phosphorylase cDNAs from muscle and liver. Restriction endonuclease designations: *Bam*HI (B), *Hin*dIII (H) and *Kpn*I (K). Ends of λlp2 and λmp1 inserts were at *Eco*RI sites. Asterisk, a sequencing reaction performed using a phosphorylase-complementary oligomer as primer.

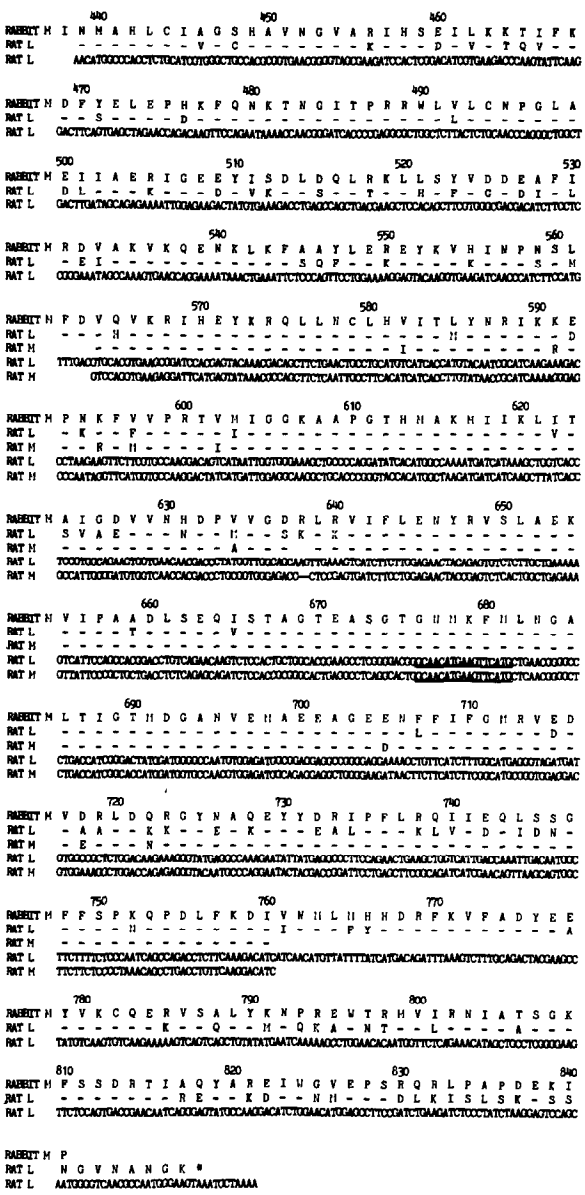

Fig.4. Partial nucleotide sequence and deduced amino acid sequence of the rat liver and muscle phosphorylase isozymes and comparison with the rabbit muscle enzyme. Amino acids in letter notation are numbered beginning with residue 440 of the rabbit muscle phosphorylase protein. Dashes, identity in amino acids for the rat liver (L) or rat muscle (M) clones with the rabbit muscle sequence; underlined sequence, site used for a phosphorylase-complementary oligomer during sequencing of the liver and muscle cDNAs; asterisk, stop codon for the rat liver cDNA.

fig.3. Fig.4 shows the determined nucleotide sequence and deduced amino acid sequence for the rat liver and muscle clones in comparison to the rabbit skeletal muscle primary sequence [1]. Based on the partial cDNA sequence and the known amino acid sequence of the rabbit skeletal muscle enzyme, several facts are evident. Comparison of the determined rat liver sequence with the rabbit muscle amino acid sequence indicates an 82% homology. The limited rabbit muscle phosphorylase nucleotide sequence information obtained by Putney et al. [32] and Hwang et al. [33] indicates a 68 and 64% homology, respectively, with the corresponding rat liver phosphorylase domain. The limited rat muscle sequence we obtained is 95% homologous with the rabbit muscle sequence. Comparison of the overlapping region sequenced for the rat liver and muscle clones shows a 79% amino acid homology and a 74% conservation at the nucleotide level. The difference between amino acid and nucleotide conservation appears to be due to a bias in codon usage between the two tissues. The majority of amio acid substitutions are predicted to have little effect on the structure and properties of the enzyme. A striking difference in the amino acid sequence between the rat liver and muscle sequences was found at the COOH-terminus. The muscle clone terminates at residue 840 (isoleucine) [33], whereas the liver clone (fig.4) has eight additional amino acids terminating with a lysine (residue 848). Furthermore, between residues 815–840, 17 of 26 amino acids (65%) differ between the liver and muscle clones. This differs from the overall 80% conservation of amino acid sequence determined for liver and muscle clones. Whether this significant deviation in amino acid sequence at the carboxy-terminus of liver and muscle phosphorylase is related to a functional difference between the two isozymes has yet to be defined.

Northern analysis shown in fig.5 indicates the size of the liver and muscle phosphorylase mRNA to be 3.1 and 3.2 kb, respectively. Based on the size of the phosphorylase mRNA and translated

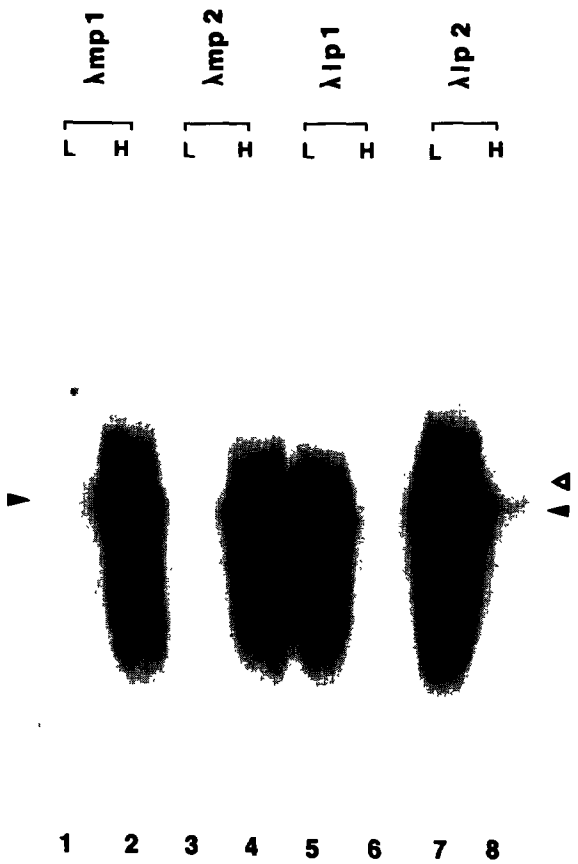

Fig.5. RNA blot of glycogen phosphorylase mRNAs. RNA was isolated from rat liver (L) and heart (H) by guanidinium isothiocyanate precipitation by the method of Chirgwin et al. [31]. Poly(A)$^+$ RNA was purified by two cycles of oligo(dT)-cellulose chromatography. Poly(A)$^+$ RNA was heated at 60°C for 5 min in 50% formamide and 6.5% formaldehyde. After addition of 10 × loading buffer (50% glycerol/10 mM EDTA/0.4% bromophenol blue/0.4% xylene cyanol) in 1/10 of the sample volume, the RNA was separated by electrophoresis on a 0.8% agarose gel containing 6% formaldehyde, 1 mM EDTA, 5 mM sodium acetate and 20 mM Mops, pH 7.0. RNA was transferred to Z-probe (Bio-Rad) according to Thomas [37]. Filters were prehybridized with 50% formamide, 5 × SSC, 5 × Denhardt's solution, 200 μg/ml salmon sperm DNA and 0.5% SDS for 8 h at 42°C. Hybridization was performed with 5 × 10^5 cpm nick-translated probe (> 10^8 cpm/μg) in 50% formamide, 5 × SSC, 1 × Denhardt's solution, 10% dextran sulfate and 20 mM sodium phosphate, pH 6.5, for 15 h at 42°C. Filters were washed 3 times with 2 × SSC and 0.1% SDS for 10 min at 24°C and then twice with 0.1 × SSC and 0.1% SDS for 10 min at 50°C. Blots were exposed to Kodak X-Omat film at −70°C overnight. Closed arrowheads indicate a 3.1 and 3.2 kb mRNA and the open arrowhead indicates a 3.4 kb mRNA.

proteins it is predicted that each mRNA contains 550–650 bases of noncoding region. In addition to the 3.1 kb liver mRNA, both the λlp1 and λlp2 clones detected a second mRNA species approx. 300 bases larger. Further analysis will be required to determine the relationship between this RNA species and the major 3.1 kb liver phosphorylase mRNA. Despite the homologies observed in the nucleotide sequences, high stringency conditions could be used with both the liver and muscle cDNA probes to measure isozyme-specific mRNA in the appropriate tissues. This finding indicates the usefulness of these probes for characterizing the expression of isozyme-specific phosphorylase mRNA in muscle and liver. Fig.6 shows the Southern analysis of rat genomic DNA using liver and muscle phosphorylase isozyme-specific probes. The differences in the intensity and pattern of hybridization for *Bam*HI, *Kpn*I and *Pst*I restricted genomic DNA indicated that at least two genes for phosphorylase are present in the rat genome. The fact that the isozymes are coded for by different genes is also indicated by the differences in nucleotide sequence for the liver and muscle clones (fig.4).

Our results indicate that the rat phosphorylase cDNA probes will be useful for studying tissue-specific isozyme expression of the key regulatory enzyme involved in glycogen breakdown. The significant conservation of phosphorylase sequences between rat isozymes, the rabbit skeletal muscle enzyme [32,33] and phosphorylases characterized from other species [34] suggests that the expression of the phosphorylase isozyme genes is related to tissue-specific gene expression mechanisms and probably not for major differences in the regulatory properties of the enzyme in liver and muscle. The similarities in allosteric regulation of the enzyme in muscle and liver support this hypothesis. Our studies take on added importance since glycogen synthase, the regulated enzyme in glycogen synthesis, also exists as isozyme forms in muscle and liver [35]. We are presently isolating the cDNA for glycogen synthase to examine the regulation of expression of the two glycogen metabolizing enzymes in muscle and liver. Other isozyme systems such as creatine kinase have also been shown to have very closely related sequences [36], similar to our findings of tissue-specific transcriptional regulation of genes coding for highly conserved phosphorylase enzymes. Availability of suitable cDNA probes for phosphorylase allows us to begin to address this problem for the enzymes involved in glycogen metabolism and how this differential expression is controlled in development and altered in the diabetic animal.

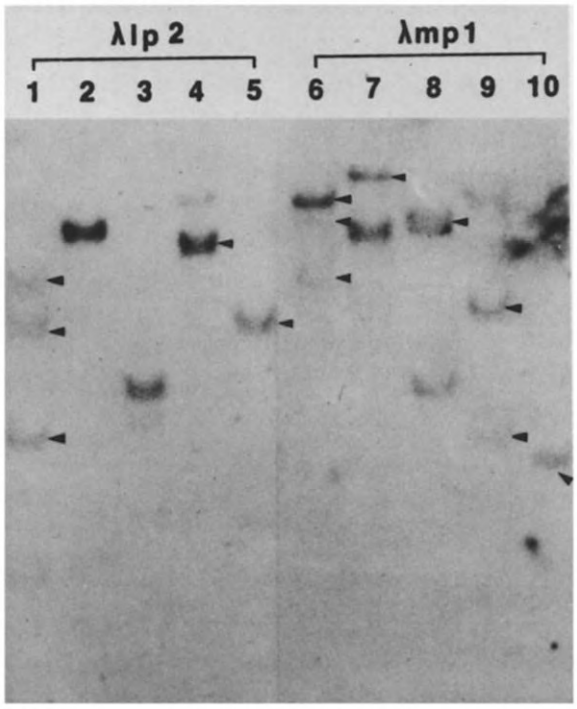

Fig.6. Hybridization of rat liver (λlp2) and muscle (λmp1) probes to restriction digests of rat genomic DNA. High molecular mass genomic DNA was prepared from rat hepatocytes by the method of Gross-Bellard et al. [38]. The genomic DNA was digested with *Bam*HI (lanes 1 and 6), *Eco*RI (lanes 2 and 7), *Hin*dIII (lanes 3 and 8), *Kpn*I (lanes 4 and 9) or *Pst*I (lanes 5 and 10) size-fractionated on a 0.7% agarose gel and transferred to Z-probe. The filters were prehybridized for 6 h at 65°C in 6 × SSC, 0.6% SDS, 5 × Denhardt's solution and 100 μg/ml salmon sperm DNA. The Southern genomic blot was hybridized with 10^6 cpm/ml nick-translated DNA probe for 15 h at 42°C in the same hybridization buffer described in fig.5 for the RNA blot. Filters were then washed twice with 2 × SSC and 0.1% SDS for 1 h at 24°C and then 3 times with 0.2 × SSC and 0.1% SDS for 30 min at 55°C. Arrows indicate differences in hybridization with the λlp2 liver and λmp1 muscle clones.

ACKNOWLEDGEMENTS

We gratefully acknowledge the helpful discussion of Chee-Wai Woon, Ellen Weiss and Jill Rulfs during the preparation of this manuscript. Supported by NIH grants GM30324, NS18779, HL20476, AM18269, 3416, 31116 and the Diabetes-Endocrinology Research Center at the University of Massachusetts Medical Center. G.L.J. is an Established Investigator of the American Heart Association.

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