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Research Article

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Molecular Mechanisms of McArdle's Disease (Muscle Glycogen Phosphorylase Deficiency)

RNA and DNA Analysis

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Abstract

Lack of muscle glycogen phosphorylase activity leads to McArdle's disease, a rare metabolic myopathy. To investigate its molecular basis at the nucleic acid level, we isolated muscle phosphorylase cDNA clones from a human cDNA library in *Escherichia coli* plasmid pBR 322. Subcloning of one insertion of M13 bacteriophage permitted its definite identification by sequencing. Northern blot experiments revealed one specific messenger RNA of 3.4 kilobases found uniquely in tissues expressing muscle phosphorylase.

We show that McArdle's disease exhibits a molecular heterogeneity at the messenger RNA level. In eight unrelated cases of McArdle's disease in which no inactive proteins had been detected, we assayed muscle biopsies for phosphorylase mRNA by Northern blotting. In five cases, no muscle phosphorylase mRNA could be detected, while in three other cases, normal length mRNA was present in lower amounts.

Moreover, Southern blot analysis of DNA isolated from white blood cells in four McArdle patients revealed no major deletion or rearrangements of the phosphorylase gene as compared with controls.

Introduction

McArdle's disease is a rare form of metabolic myopathy, genetically transmitted as a recessive autosomal trait.

Clinically, this disease is characterized by asthenia, cramps, stiffness, and attacks of myoglobinuria following physical effort. It is now well established that these manifestations of the disorder originate from a depletion in the ATP stores, needed for muscle contraction and relaxation, induced by the absence of glycogen phosphorylase enzyme activity. This enzyme catalyzes the first step of the glycogenolytic pathway that provides most of the readily available energy for intensive muscle contraction. In patients with McArdle's disease, it has been demonstrated by Mommaerts et al. (1) and Schmid and Mahler (2) that glycogen phosphorylase activity is deficient.

In human tissues and those of other mammals, glycogen phosphorylase exists in three isoenzymatic forms: liver, muscle, and brain (3). The muscle form is the only isoenzyme expressed in skeletal muscle and is also found in heart and brain along with the cerebral form. This explains why the defect in McArdle's disease seems confined to muscle tissue.

The first investigations of the molecular basis of McArdle's disease consisted of the search for an inactive protein in patients' muscle. Two kinds of results were obtained: in rare cases, an immunological cross-reacting material was detected in variable amounts (4, 5), but most patients showed little or no cross-reacting material (CRM)[1]. This biochemical heterogeneity can be confirmed by direct visualization of the phosphorylase protein with one- (5) or two-dimensional (6) electrophoresis of the muscle extracts.

Subsequently, the presence of muscle phosphorylase RNA-translational activity was investigated in our laboratory in two patients without detectable cross-reacting material and in two heterozygous relatives. It was found that the absence of phosphorylase protein was due to the lack of functional messenger RNA (mRNA) (7).

Our goal in undertaking the work presented here was to further characterize the molecular basis of McArdle's disease through direct detection of phosphorylase mRNA and investigation of the phosphorylase gene with specific complementary DNA (cDNA) probes.

Methods

Materials. Usual chemicals of the highest purity available were from Merck, Bracco, S.p.A., Milan, Italy, Sigma Chemical Co., St. Louis, MO, Serva Fine Biochemicals, Inc., Garden City Park, NY, and Boehringer Mannheim Biochemicals, Indianapolis, IN. "Gene screen plus" nylon filters were from New England Nuclear, Boston, MA, [α^{32}P]deoxycytidine triphosphate and [α^{35}S]deoxyadenine triphosphate were from Amersham Corp., Arlington Heights, IL, and methylmercuryl hydroxide was from Ventron Corp., Beverly, MA. Reverse transcriptase was from Genofit, DNA polymerase large fragment according to Klenow and restriction enzymes were from Amersham Corp., and Kornberg DNA polymerase I was from Boehringer Mannheim Biochemicals.

Human material. Muscle biopsies were available from eight unrelated patients with McArdle disease (patients 2, 3, 5–10) and from two previously studied heterozygotes (H_1 and H_2). Control muscle came from patients suspected of muscle disease but found normal after biopsy examination. Blood samples could be obtained from only two of these patients (2, 3) and from two additional patients (1, 4) from whom no biopsy sample was available. All patients are Caucasian and from different regions of France. In all cases, informed consent was obtained and research was carried out in conformity with the declaration of Helsinki.

Enzymatic and protein assay. The diagnosis of McArdle's disease was established according to histochemical (8), biochemical (9), and clinical criteria. All muscle samples were screened for phosphorylase protein by analysis of soluble muscle proteins by denaturing polyacrylamide gel electrophoresis (PAGE) according to Laemmli (10).

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1. *Abbreviations used in this paper:* CRM, cross-reacting material; 1× SSC, 0.15 M NaCl + 0.015 M sodium citrate.

cDNA library screening. ~8,000 clones of a human muscle cDNA (11) library were screened at low stringency with a 100-basepair (bp) rabbit muscle phosphorylase cDNA probe kindly given by Dr. Putney (Department of Biology, Massachusetts Institute of Technology) (12). Thus we detected three cross-hybridizing cDNA clones (18F11; 20ph; 19ph) containing insertions ~ 400, 500, and 1,300 bp long, respectively.

Localization of these clones was achieved by partial-restriction enzyme mapping (13). Two PstI–PstI fragments of the longer clone 19ph, ~600 and 700 bp long, were subcloned in both orientations at the PstI site of mp10 M13 phage (14) for use as probes. Subclones of these fragments were sequenced on both strands with the Sanger method of dideoxynucleotide termination (15). Human aldolase A cDNA clones were obtained and subcloned in M13 phage as described elsewhere (16).

Labeling of probes. Preparation of high radioactive activity–specific single-stranded probes was performed by a modification of the techniques first described by Messing et al. (14) and Church et al. (17). Extension of M13 universal synthetic primer by the Klenow fragment of DNA polymerase I permitted labeling of the cDNA fragments subcloned in vector phage M13. Denaturation of the newly polymerized, labeled fragment and M13 DNA matrix was performed by adding 0.2 vol of a mixture of 250 mM NaOH, 5 mM EDTA, 50% glycerol and bromophenol blue boiling for 3 min, then submitting to electrophoresis in a 1.2% low-melting agarose, 50 mM NaCl, 1 mM EDTA gel equilibrated in migration solution, 30 mM NaOH, 1 mM EDTA, for 40 min. Migration for 2 h at ~4 V/cm permitted isolation of the labeled probe, which was cut off after visualization by autoradiography. Agarose fragments were then directly added to hybridization mix and melted by boiling for 15 min. This method usually yielded specific activities of 2×10^9 cpm/μg of probe.

RNA purification. Total RNA was prepared from muscle biopsies weighing 30–80 mg with a modification of the method previously described (18). Samples were homogenized in the guanidium chloride/sodium dodecyl sulfate (SDS) buffer with a Potter–Elvehjem homogenizer. To improve the yield of RNA recovery from such a small amount of RNA-poor tissue, 50 μg of carrier *Escherichia coli* ribosomal RNA was added to the homogenate before the first ethanol precipitation. Abundance and integrity of recovered RNA were checked by electrophoresis and estimations of both the amount of ribosomal RNA and the 28S/18S ratio.

Northern blot analysis. RNA samples were equalized to 20 μg/slot by addition of *E. coli* ribosomal RNA, then run on a vertical methyl mercury hydroxide–1.5% agarose gel. After transfer to nylon filters as described (19), hybridization was performed for 40 h at 65°C in a 3× standard saline citrate (SSC) solution containing 0.1% polyvinyl pyrrolidone, 0.1% glycine, 0.1% Ficoll-Hypaque, 10% dextran sulfate, 1% SDS, 10 μg/ml poly A, 100 μg/ml salmon sperm DNA, plus 1–3 million cpm/ml of both M13 probes (termed "600" and "700"), with 0.5 ml of hybridization mix per cm^2 of filter. Final washing was performed in 0.2× SSC + 1% SDS at 65°C. Aldolase A mRNA is 1,550 nucleotides long. Its relative amount is unchanged in various normal muscle samples and McArdle's disease biopsies, as shown by comparison with 28S and 18S ribosomal RNA signals (not shown), obtained by hybridization of filters with a cDNA probe complementary to testis polyA(−)RNA (20). It thus constituted a useful internal standard for quantification of mRNA. Phosphorylase and aldolase A mRNAs were probed on the same filter that was then autoradiographed overnight at −80°C on AR–XOmat films (Eastman Kodak Co., Rochester, NY) with intensifying screens.

mRNA length was estimated by comparison with migration standards (*E. coli* 23S and 16S, human 28S and 18S ribosomal RNAs). The relative intensity of both bands was measured by densitometric scanning of the autoradiograms with a densitometer (Shimadzu Seisakusho Ltd., Kyoto, Japan).

Southern blot analysis of genomic DNA. Total human genomic DNA was prepared from white blood cells as reported (21). Southern blotting was performed with 2–5 μg of total genomic DNA as described (22). DNA was cut by restriction enzymes Bam HI, BglI, BglII, Eco RI, and Hind III, and submitted to electrophoresis on an 0.8% agarose gel for 16 h at 30 mA. The gel was bathed 5 min in 0.25 M HCl, then DNA was denatured by twice putting the gel in a 0.4 M NaOH, 0.6 M NaCl solution for 10 min, followed by transfer to a nylon membrane in the same solution. Filters were neutralized by two 15-min washes in a 0.5 M Tris HCl, 1 M NaCl buffer (pH, 7) hybridized successively with the two phosphorylase probes (first 700, then 600). Washes and autoradiography were done as if for Northern blots.

Results

Restriction mapping permitted location of the three muscle phosphorylase cDNA inserts in relation to the rabbit cDNA insert used for screening. All three insertions covered a COOH terminal region of the phosphorylase cDNA (Fig. 1).

Identification of the cDNA clones by nucleotide sequencing. The longest, most informative clone, (19ph), spans about half the coding region of the cDNA and a short portion of the 3′ noncoding region (Fig. 2). This insertion was subcloned in bacteriophage vector M13. Its sequencing by the Sanger dideoxy technique showed a 94% homology between the deduced amino acid sequence and that of the rabbit protein (23). Recently, Hwang et al. (24) have characterized a human phosphorylase cDNA clone that overlaps clone 19ph and extends further into the 3′ noncoding region, whereas 19ph cDNA extends more toward the 5′ end (Fig. 1). Of the 1,300 bp we sequenced, we report here a 658-bp region lying upstream of that reported by Hwang et al. (Fig. 2). Comparing nucleotide sequences of rabbit and human cDNAs, they reported a 90% homology. The same homology can be noted in the overlapping region between the human 19ph and the rabbit cDNA they described (24).

Expression of muscle phosphorylase mRNA in different normal human tissues. Northern blot analysis of various adult and fetal human tissues detected a 3.4-kilobase (kb) mRNA species in adult muscle that was not detectable in liver and brain of a 3-mo-old fetus or in adult liver and lung (Fig. 3). Such a mRNA length is compatible with that expected for a messenger encoding a protein of about 841 amino acids, which is the length of rabbit muscle glycogen phosphorylase (23). This indicates that noncoding sequences consist of ~800–900 bp, including the poly A tail.

Protein analysis of McArdle patients. Phosphorylase enzyme activity was undetectable in all 10 patients, and no protein band corresponding to the normal phosphorylase position as seen in controls could be detected by denaturing one-dimensional PAGE (not shown).

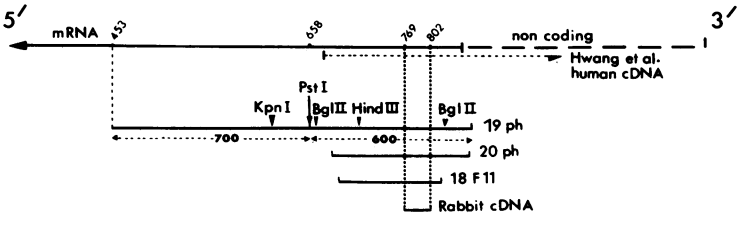

Figure 1. Partial restriction map and localizations of three muscle glycogen phosphorylase cDNA insertions with respect to phosphorylase messenger RNA. The extent of the small rabbit cDNA probe used for screening, and of the Hwang et al. cDNA insert are also shown.

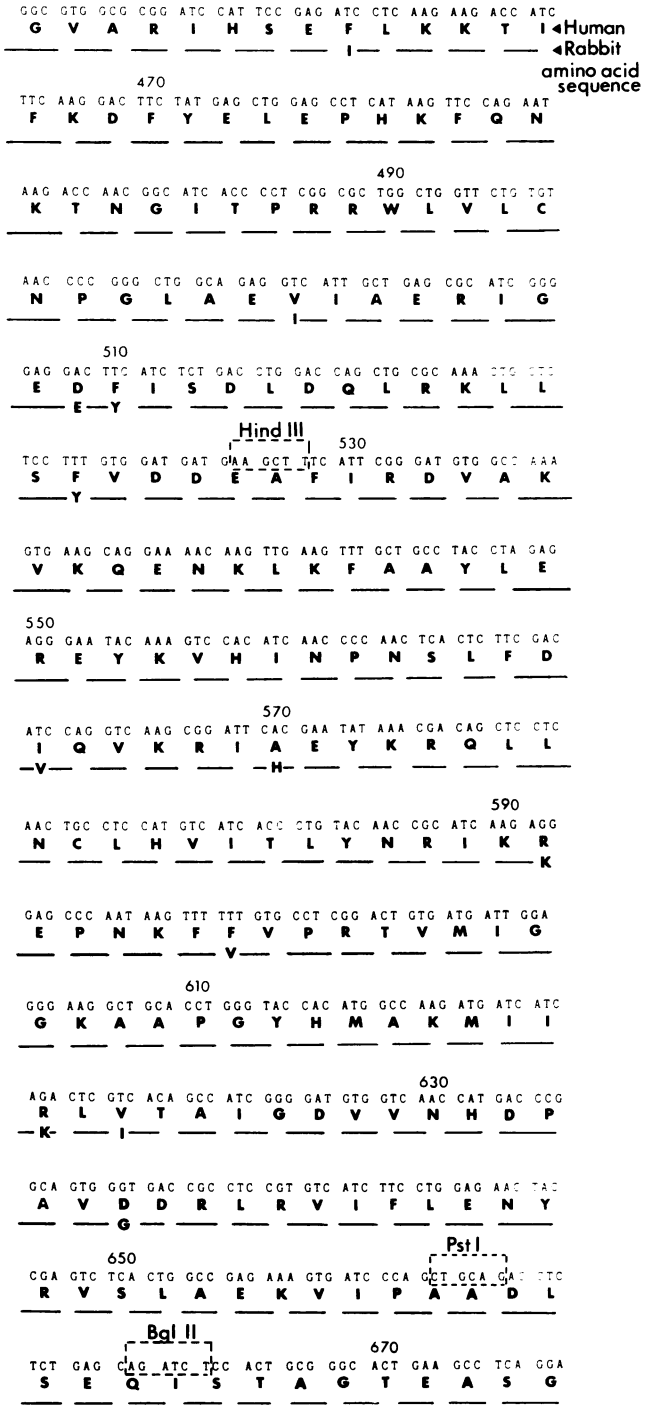

Figure 2. Partial nucleotide sequence of human glycogen phosphorylase cDNA insertion (19ph) and comparison of deduced protein sequence with that of rabbit muscle glycogen phosphorylase protein. Corresponding amino acids are given under each nucleotide triplet, as well as aminoacids that differ in the rabbit cDNA sequence.

Northern blot analysis of RNA. Muscle biopsies weighing 30–80 mg were available from eight patients. They yielded a total RNA recovery of 5–20 μg per biopsy as roughly estimated from the intensity of 18S and 28S ribosomal RNA bands visualized on agarose gel.

On Northern blots of normal muscle RNA, the single-stranded probes enabled us to detect phosphorylase mRNA

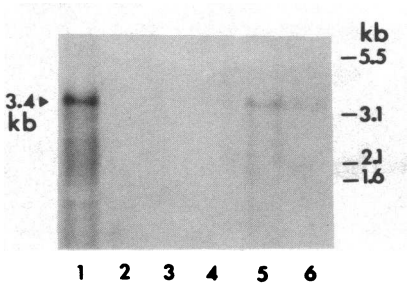

Figure 3. Northern blot analysis of normal human fetal and adult tissue RNA with 18F11 phosphorylase cDNA probe. (*1*) adult muscle, 2 μg of total RNA; (*2*) adult liver, 10 μg of total RNA; (*3*) fetal brain, 20 μg of total RNA; (*4*) lung, 20 μg of total RNA; (*5*) adult muscle, 0.5 μg of total RNA; (*6*) fetal muscle, 5 μg of total RNA.

starting with only 0.5 μg of total RNA deposited on the gel, i.e., ~50 pg of specific RNA.

Next, Northern blots of patients' muscle RNA were hybridized with both phosphorylase and aldolase A probes (Fig. 4). In five patients (2, 5, 6, 9, and 10) no specific phosphorylase mRNA could be seen, whereas aldolase A mRNA was present.

In the other three patients, an apparently normal-sized messenger RNA was revealed, but its amount, appreciated from the ratio aldolase signal/phosphorylase signal (Table I), was decreased.

In addition, for the two heterozygotes (H_1 and H_2) in which we had previously demonstrated a decrease of functional mRNA (7), hybridizable phosphorylase mRNA was reduced in a parallel fashion to ~50%.

Southern blot analysis of genomic DNA. Southern blot experiments were carried out on genomic DNA prepared from white blood cells of four patients and normal individuals.

One to three restriction fragments hybridized strongly with the different phosphorylase probes. This permitted, by combining results obtained from the five types of digestion used, the scanning of about 25 kb of DNA, including the C terminal half of the gene. For all individuals studied, restriction patterns were similar in patients and normal controls (4–10 according to the enzyme) (Fig. 5, *a* and *b*). It should be noted that two of these patients were also analyzed by Northern blot. One was found to lack specific mRNA (patient 2); the other exhibited a diminished amount of phosphorylase mRNA (patient 3). Moreover, patient 4 is the son of heterozygote H_2, in which we demonstrated a 50% reduction of muscle phosphorylase mRNA.

Thus, three patients were evaluated, both at the DNA level, where no anomaly was found, and the RNA level with patient 2 and heterozygote H_2 corresponding to "nonexpressing alleles" and one, patient 3, corresponding to a "diminished RNA" pattern.

Discussion

By heterologous hybridization with a small rabbit muscle glycogen phosphorylase cDNA probe, we isolated three human glycogen phosphorylase cDNA clones from a human muscle cDNA library.

A firm identification of these clones was obtained by sequencing these insertions, thus determining their position in respect to the known rabbit protein sequence. All three were located in the C terminal half of the mRNA, with the longer insert (19ph) covering two fifths of the total messenger and half of the coding sequence.

Comparison of amino acid sequence deduced from a cDNA sequence of the 19ph insert with the rabbit protein sequence

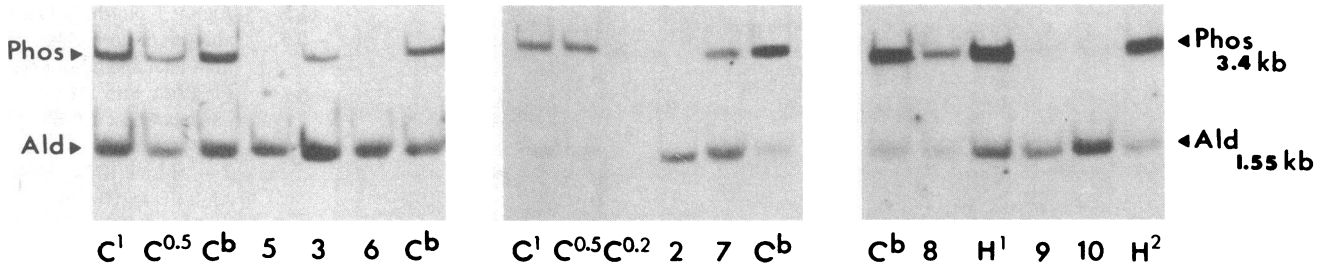

Figure 4. Northern blot experiment. McArdle and control mRNA were hybridized simultaneously with both 600 and 700 M13 phosphorylase probes and M13 aldolase probes. Controls consisted of either known amounts of total RNA prepared in standard conditions (11) from human muscle or total RNA from normal human biopsies prepared parallel to the McArdle biopsies. (C^1) Control, 1 μg of total muscle RNA; ($C^{0.5}$) control, 0.5 μg of total muscle RNA; ($C^{0.2}$) control, 0.2 μg of total muscle RNA; (C^b) control, normal biopsy; (*1 to 10*) McArdle patients, (H_1 *and* H_2) heterozygote cases. The exact amount of muscle RNA blotted onto the filter could not be indicated for all biopsy RNAs, as discussed in the text. It probably varied from 1 to 10 μg. Apparent phosphorylase/aldolase ratio on autoradiograms varies from one experiment to another depending on the relative specific activities of phosphorylase and aldolase M13 probes.

showed a 94% homology between the two protein sequences. Used as probe in Northern blot analysis, the insert recognized a single specific RNA species of 3.4 kb, that was detected only in skeletal muscle, the only tissue among those examined known to express muscle-type glycogen phosphorylase (3).

In contrast, the probe didn't hybridize in the stringency conditions we used with any brain or liver messenger, thus indicating that it was specific for the muscle isozymes' RNA and did not recognize the brain and liver isozymes' RNA.

Because of the small size and low RNA content of muscle biopsies, we then focused on improving the methods. We first modified classical RNA purification techniques to adapt to small muscle samples. In addition, we used efficient, highly labeled single-stranded cDNA probes copied from subclones in the M13 vector phage that also permitted easy Southern blot analysis of as little as 2 μg of genomic DNA.

Purification of muscle RNA from small muscle biopsies was made possible by the use of *E. coli* ribosomal RNA as carrier. The inconvenience of this technique was that it precluded any easy determination of eukaryotic RNA in the obtained preparations and then any direct determination of phosphorylase mRNA from Northern blot analysis. Therefore, we needed to use an internal standard representative of the amount of muscle RNA really transferred onto the filters. Aldolase A mRNA appeared to be a convenient standard. Its amount appreciated with respect to total muscle RNA and to the intensity of 28S and 18S ribosomal RNA bands hybridized on the blots (20), relatively constant in different samples of normal deltoid and quadriceps muscles, and the phosphorylase/aldolase ratio in these tissues was identical in each experiment. Although the relative intensity of the phosphorylase and aldolase mRNA bands varied for different experiments due to differences in specific and total radioactivity of each probe, phosphorylase mRNA concentration for a given experiment could be correctly evaluated with respect to normal controls blotted on the same filter. It was given by the ratio of phosphorylase band/aldolase band in the patient versus phosphorylase band/aldolase band in the controls.

Table I. Quantification of Phosphorylase mRNA

Case	2	3	5	6	7	8	9	10	H1	H2
Percent	0	33	0	0	16	34	0	0	45	48

Relative abundance of phosphorylase mRNA in McArdle patients muscle. A single experiment was performed for each patient and for heterozygote cases. Each phosphorylase value obtained by scanning is normalized by dividing it by the aldolase A value for the same patient. This ratio is then expressed as a percentage of the average phosphorylase/aldolase ratio measured in control muscle RNA and normal biopsies on the same filter (5–8 controls of both types per filter).

The major fact arising from the investigation of eight unrelated cases with no CRM is that in some patients' muscle no specific glycogen phosphorylase mRNA is detected, whereas in others apparently normal sized mRNA is present, but in diminished amounts. These results contrast with most of the other human genetic diseases presently characterized: citrullinaemia (25), Lesch–Nyhan syndrome (26), phenylketonuria (27), Sandhoff's disease (28), Tay–Sachs disease (29), and adenosine deaminase (30) and phosphoglycerate kinase deficiencies (31), in which relatively few cases show diminished amounts of mRNA, and at least some patients in each study exhibit quantitatively normal amounts of specific RNAs. It seems probable that this discrepancy partly arises from the fact that patients with cross-reacting protein material are included in most of these studies. This is not the case for us. On the other hand, our observation of two distinct mechanisms suggests that some McArdle patients could be compound heterozygotes. In particular, those with mRNA levels of 20–30% of normal might either be true homozygotes or compound heterozygotes with one allele expressing no mRNA at all, whereas the other could express ~40–60% of the usual level. Our results clearly demonstrate a heterogeneity at the mRNA level in the mechanisms leading to McArdle's disease, as previously documented for protein expression. Hence, three different molecular types have been defined so far in McArdle's disease, characterized by (*a*) presence of an inactive protein, (*b*) absence of protein and presence of nonfunctional mRNA in decreased amounts, and (*c*) absence of both protein and mRNA.

Southern blot analysis reveals only a small number of restriction fragments with each phosphorylase probe. This is compatible with the existence of one unique gene, localized by chromosome hybridization experiments on chromosome 11 (32). Moreover, for the five enzymes tested, we find no evidence for frequent polymorphic restriction sites among the samples analyzed (8–14 individuals, both controls and patients, according to enzyme).

Recent studies (Anderson, L., Fourth International Congress on Neuromuscular Diseases, 6–11 July 1986, Los Angeles, CA)

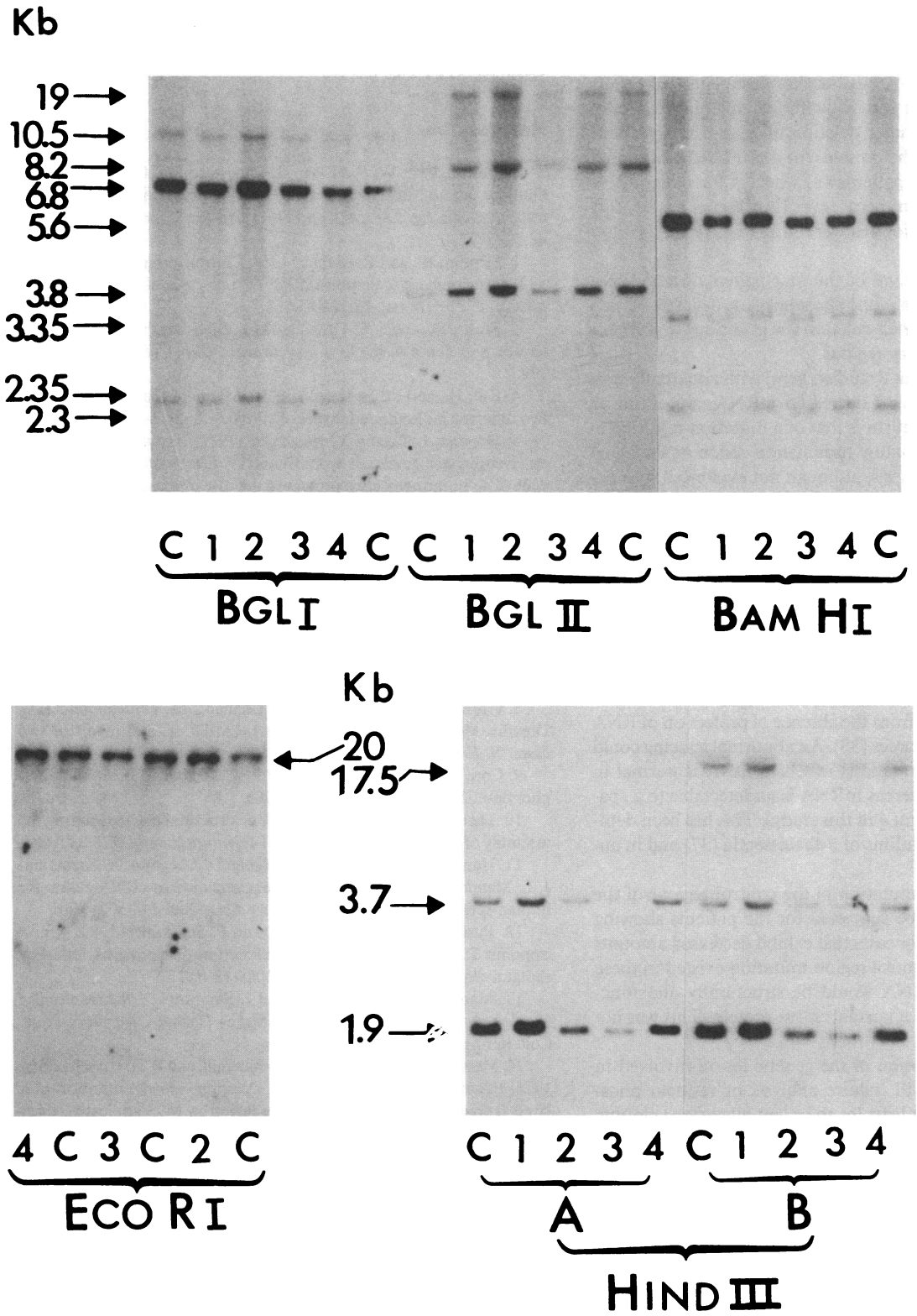

Figure 5. (*Top*) Southern blot analysis of McArdle patient and control DNA with restriction enzymes BglII, BglI, and Bam HI. 2 μg of DNA were run per slot. Hybridization was performed with M13 probes 600 and 700. (C) Control DNA; (*1, 2, 3, 4*) MacArdle patient DNA. (*Bottom*) Southern blot analysis of McArdle patient and control DNA with restriction enzymes Eco RI and Hind III. 2 μg of DNA were run per slot. Hind III-A was hybridized with 700, the most 5′ probe, whereas Eco RI and Hind III-B were hybridized with both M13 probes 600 and 700. (*C*) Control DNA; (*1, 2, 3, 4*) McArdle patient DNA.

show that human muscle glycogen phosphorylase is encoded by a relatively small gene of ~13 kb. Because the DNA fragments revealed in Southern blot analysis span ~25 kb, we think we have explored the totality of the phosphorylase gene. Our results, obtained in four different patients with five different restriction enzymes, preclude any major deletion or gene rearrangement. This is compatible with the presence of diminished amounts of mRNA for one of these patients (3) whereas for patient 2 no conclusions can be drawn, because mRNA seems absent. Unfortunately, no muscle biopsies were available for the last two cases.

Therefore it appears that for the four patients whose DNA was studied here, the origin of the phosphorylase deficiency could be a point mutation or small deletion (or insertion) in the gene or in its flanking regulatory regions.

In patients (e.g., patient 3 studied here) with apparently normal phosphorylase gene and decreased mRNA concentration the most plausible mechanism is that of a mutation resulting in either the advent of a premature termination codon or abnormal splicing. The latter hypothesis, although not evidenced by a detectable size abnormality of phosphorylase mRNA in patients 3, 7, and 8, cannot be ruled out, as is well illustrated in patients with citrullinemia, in which abnormal arginosuccinate synthetase mRNAs with normal size appear to be frequent (25). The decrease of the mutant mRNA concentration could arise from instability of the messengers carrying an early nonsense mutation due to the occurrence of a stop codon in an exon or an unspliced intron fragment as already described in different types of thalassemia (33–37). In thalassemia it has been proposed that such an instability could result from the absence of protection of RNA by its integration in polysomes (33). An abnormal splicing could also account for the observations in which DNA is normal in Southern blot analysis whereas mRNA is undetectable (e.g., patient 2 and probably patient 4 in this study). This has been demonstrated in some observations of β-thalassemia (37) and in analbuminemic rats (38).

The eventuality of a mutation in the control regions of the phosphorylase gene can be suggested for the patients showing no mRNA at all, but not for cases that exhibit decreased amounts of mRNA. Indeed, if a control region mutation existed in these latter cases, residual mRNA would be structurally and functionally normal and yield a normal active protein. This was not the case.

The definitive elucidation of the genetic lesion involved in the enzyme deficiency will require analysis of residual phosphorylase mRNA, and, at least for the silent alleles, the cloning of the mutant genes and detailed analysis of their nucleotide sequences.

In conclusion, this study demonstrates that in four patients with muscle phosphorylase deficiency, the phosphorylase gene was present and apparently not rearranged. However, a clear heterogeneity was proved at the mRNA level. Phosphorylase mRNA was undetectable in five patients, and in three patients it was present in decreased amounts but with a normal length.

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