AN INVESTIGATION OF NUCLEAR-CYTOPLASMIC RELATIONSHIPS IN SKELETAL MUSCLE FIBRES OF MOUSE CHIMAERAS

By

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ABSTRACT

Mature mammalian myofibres are multinucleated elongate cells in which the nuclei are regularly distributed along the entire length of the cell. The multinucleated condition arises by the fusion of mononucleated myoblasts to form myotubes during primary development. A fundamental and essentially unexplored problem in muscle cell biology is the question of the nuclear-cytoplasmic relationship in muscle fibres. Although local membrane specializations occur, most notably in the region of innervation, it is not known if each myonucleus controls the biochemical functions of a local territory comprised of the immediately adjacent muscle cytoplasm, organelles, and membrane, or if the gene products of every myonucleus are uniformly distributed in the fibre. An understanding of this aspect of the organization of muscle cells could help solve other problems in the biology of skeletal muscle; for example, what is the significance of X-chromosome mosaicism in the expression of muscle disease in carriers of X-linked myopathies? Which myonuclei are the targets of neurotrophic influences on muscle?

In this study I have taken advantage of the fact that in mouse chimaeras two genotypically distinct types of myonuclei are often incorporated into single myofibres, in order to investigate how multiple myonuclei maintain skeletal muscle cells *in vivo*. Mouse chimaeras were produced by the aggregation of embryos homozygous for different alleles for both the monomers of the cytoplasmic enzyme glucosephosphate isomerase (GPI-1) and the monomers of the nuclear-coded mitochondrial matrix protein malic enzyme (MOD-2). Therefore, mosaic myofibres contained both $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^a}$, $\underline{Mod\text{-}2^a}/\underline{Mod\text{-}2^a}$ myonuclei and $\underline{Gpi\text{-}1^b}/\underline{Gpi\text{-}1^b}$, $\underline{Mod\text{-}2^b}/\underline{Mod\text{-}2^b}$ myonuclei.

The distribution of the electrophoretic variants of GPI-1 was measured at various points along the length of mosaic muscle fibres. Changes in the isozyme composition along the length of a cell would indicate that the enzyme remains localized in cytoplasmic territories. A uniform isozyme profile would result if the products of individual myonuclei are homogeneously distributed. The results presented here indicate that the intracellular distribution of GPI-1 isozymes does not change along the length of mosaic myofibres in both adult and young mouse chimaeras. Therefore, the products of each myonucleus are widely distributed in skeletal myofibres.

Functional GPI-1 is an oligomeric enzyme composed of two monomers. Analyses of the proportions of GPI-1 dimer types in mosaic myofibres reveal that the homogeneous distributions of GPI-1 isozymes arise by the widespread distribution of precursors of GPI-1 (monomers or monomer precursors such as messenger RNA) before dimer formation; dimers are not initially assembled in local territories around each myonucleus and then widely distributed.

The investigation was extended to the question of whether individual mitochondrion in skeletal myofibres have access to nuclear-coded proteins encoded by one or by multiple myonuclei. Evidence was obtained that indicates that both types of monomers of nuclear-coded mitochondrial malic enzyme (MOD-2) are present in an individual mitochondrion of a mosaic myofibre.

The results indicate that the gene products of different myonuclei are widely distributed both before the assembly of cytoplasmic oligomeric proteins and before the incorporation of proteins into mitochondria.

Taken together, the results obtained in this study constitute direct evidence that mammalian skeletal myofibres are functional syncitia in which the gene products of many myonuclei are distributed throughout each cell. Problems and possibilities regarding the mechanisms of distribution and the nature of the gene products distributed are discussed,

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LIST OF ABBREVIATIONS

nm	nanometer
µm	micrometer
mm	millimeter
cm	centimeter
mg	milligram
g	gram
kg	kilogram
sec	second
min	minute
hr	hour
wk	week
mo	month
yr	year
ml	milliliter
l	liter
V	volt
µF	microfarad

INTRODUCTION

INTRODUCTION

Skeletal muscle is a widely used model system for studying cell differentiation and cell communication. Many of the quantitative and qualitative changes in protein synthesis that occur during myogenesis have been described (for reviews see Merlie, Buckingham and Whalen, 1977; Garrels, 1979). It is known, too, that innervation exerts a major influence on gene expression in muscle cells, and investigation of the important neural factors is a major research area (see reviews by Guth, 1968; Harris, 1974; Guthmann, 1976; Purves, 1977). Skeletal muscle is also widely studied with the aim of determining the mechanisms of the many diseases of human and animal muscle (Walton, 1974; Adams, 1975; McComas, 1977).

A fundamental and essentially unexplored problem in muscle cell biology is the question of the nuclear-cytoplasmic relationship in muscle cells. Vertebrate skeletal myofibres are multinucleated cells in which the myonuclei are uniformly distributed along the length of the fibre (Fischman, 1972; Landing, Dixon and Wells, 1974). In electron micrographs, all myonuclei are similar in appearance (Schultz, 1976) and, therefore, it is generally believed that all nuclei are functionally equivalent. However, the extent of the influence of each nucleus is not known. Specifically, it is not known whether the gene products of all the myonuclei enter a common pool that is distributed throughout the fibre, or if each nucleus contributes gene products primarily to the local cytoplasm, organelles and membrane.

INTRACELLULAR ORGANIZATION

The investigation of how multiple nuclei maintain a skeletal muscle fibre could provide insights into several important biological problems. For example, the skeletal muscle fibre provides a unique opportunity to investigate certain aspects of intracellular organization.

Many of the subcellular elements that are common to many types of cells are identified in Figure 1. The early view of the typical cell as a membrane-bound random mixture of enzymes, metabolites and organelles in water has been replaced by the concept of the cell as a highly organized structure. The revised view of the cell is based largely on the discoveries that many cell components are precisely localized within the cytoplasm.

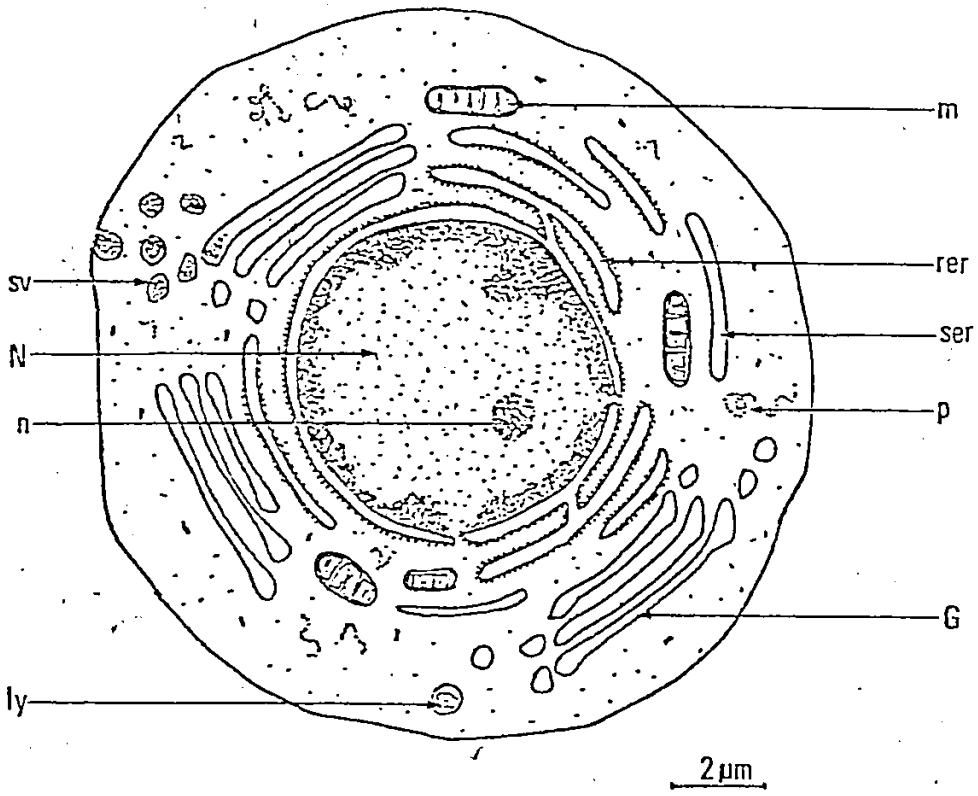
Ultrastructural studies have revealed that there are elaborate systems of cytoplasmic fibres (microtubules, intermediate or 10nm filaments, microfilaments, and 3-6nm cytoplasmic strands) in various cell types. Microtubules appear to function in generating force and providing direction for precise intracellular movements of various organelles in cultured cells (Wang and Goldman, 1978; Wang, Cross and Choppin, 1979). A role has also been described for microtubules in the transport of organelles and proteins along the axons of neurons (Jeffrey et al., 1972; Ochs, 1974). Five major classes of intermediate filaments have been biochemically and immunologically distinguished. The functions of these cytoskeletal elements are thought to include provision of mechanical support and maintenance of nuclei and other cell organelles at their characteristic positions in cells (reviewed by Lazarides, 1980). Bundles of actin-containing microfilaments function both as structural supports in extended cell processes and as part of the actomyosin-based

Figure 1

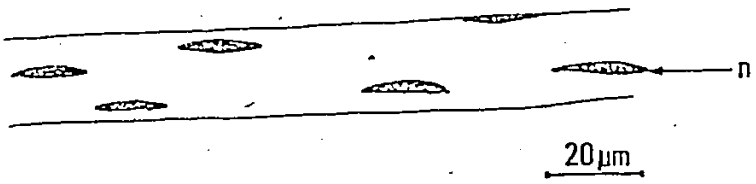
a) Diagrammatic illustration of an ideal animal cell showing the arrangement of the major organelles and the continuity between membrane-bound elements. The components include the Golgi apparatus (G), lysosomes (ly), mitochondria (m), nucleus (N), nucleolus (n), polysomes (p), rough endoplasmic reticulum (rer), smooth endoplasmic reticulum (ser), and secretory vesicles (sv), (after Porter and Bonneville, 1974.)

b) Diagram illustrating the arrangement of myonuclei in a segment of a mammalian skeletal muscle fibre (after Landing et al., 1974).

a)



b)



contractile apparatus of motile nonmuscle cells (Goldman, Milsted, Schloss, Starger and Yerna, 1979). Microfilaments are also thought to anchor some membrane bound proteins at specific sites on the plasma membrane (Rothman and Lenard, 1977). Wolosewick and Porter (1976) have presented ultrastructural evidence that 3-6nm thick strands of cytoplasmic ground substance form a three-dimensional lattice throughout the cytoplasm of cultured human WI-38 cells. They suggest that this 'microtrabecular system' is continuous with the larger fibre elements and that the entire system forms a structured frame for the nonrandom distribution of organelles such as nuclei, ribosomes, and the membranous systems of endoplasmic reticulum and Golgi apparatus.

Another characteristic of intracellular organization is that the activities of many enzymes are specifically localized in intracellular compartments (e.g. lysosomes, mitochondria, secretory vesicles) or bound to particular membranes (e.g. plasmalemma, mitochondria, endoplasmic reticulum)(Mahler and Cordes, 1971). Although 20-25% of the total protein content of a cell may be recovered in the soluble fraction of cell homogenates, there is evidence that many of these 'soluble' proteins are in fact loosely associated with other cell components in vivo. Pette and co-workers have found that several glycolytic enzymes are associated with actin filaments in rabbit skeletal muscles (Arnold and Pette, 1968; Arnold, Henning and Pette, 1971). In addition, the results of studies by Clarke and Masteris (1973) indicate that glycolytic enzymes occur as a multi-enzyme complex in rat skeletal muscle. Several groups have noted that enzymes of a given metabolic pathway are often present in a constant ratio of concentrations (e.g. Pette, Luh and Bücher, 1962), and it has been suggested that the

phenomenon of constant-proportion groups of enzymes depends on the structural arrangement of the enzymes at common sites (Lebherz, Sardo, Petell and Shackleford, 1978). These observations, combined with the teleological argument that cell components are organized for efficient and controlled function, have led some authors to question whether any enzymes at all are freely soluble in the cytoplasm (e.g. Sigel and Pette, 1969).

It has been well established that mechanisms exist for the specific localization of many newly synthesized proteins. Proteins destined for secretion are synthesized in the perinuclear region of cells on ribosomes of the rough endoplasmic reticulum (RER). The nascent proteins are discharged directly into the lumen of the RER and move along the cisternae of the RER and smooth ER to the condensing vacuoles of the Golgi complex. The proteins are then stored in membrane-bound secretory granules derived from the Golgi complex. Eventually, the proteins are discharged by exocytosis when the granule membrane fuses with the plasma membrane. Palade and colleagues originally described the process for the localization of secretory proteins in the pancreatic exocrine cells but the mechanism is now thought to be essentially identical for the transport of secretory and lysosomal proteins in many cell types (see Palade, 1975; Shore and Tata, 1977 for reviews).

There is very little known about how newly synthesized membrane proteins become associated with the "correct" cellular membranes (reviewed by Rothman and Lenard, 1977; Wickner, 1979; Davis and Tai, 1980). However, there is experimental evidence for the hypothesis that some integral membrane proteins reach the plasma membrane by a

pathway similar to that taken by secretory proteins with the exception that the nascent proteins are not released into the lumen of the ER; rather, they are incorporated into the ER membrane. Some of the evidence has come from studies of skeletal muscle. In correlated kinetic and electron microscope autoradiographic studies of newly synthesized acetylcholine receptors (AChR) in cultured chick skeletal muscle cells, Fambrough and Devreotes (1978) showed that the newly synthesized receptors are located in the perinuclear area and Golgi apparatus before they appear on the cell surface. In addition, it appears that Ca^{++} Mg^{++}ATPase, the major integral membrane protein of the sarcoplasmic reticulum (SR) is synthesized on polyribosomes attached to the SR in both cultured rat (Greenway and MacLennan, 1978) and embryonic chick (Chyn, Martonosi, Morimoto, and Sabatini, 1979) skeletal muscle.

There are also morphological and biochemical observations consistent with the theory that nuclear-coded mitochondrial proteins are synthesized on RER which may be continuous or closely associated with the outer mitochondrial membrane (Shore and Tata, 1977).

On the basis of many of these observations, Shore and Tata, (1977) have speculated that "there is a tendency for cells to compartmentalize and concentrate the synthesis of particular proteins at or near their site of action." Although neither the site of synthesis nor the final intracellular location is known for the majority of cell proteins, the possibilities emerge 1) that most proteins are not in free solution inside cells, 2) that most proteins become localized at specific sites during or shortly after their synthesis, and 3) that

these properties may be characteristic of multinucleated as well as mononucleated cells. Investigation of the extent of influence of single myonuclei in a skeletal muscle cell could provide additional information about the organization of protein synthesis and localization in multinucleated cells. If each nucleus in a muscle cell controls only the local cytoplasm, organelles and plasma membrane, then it could be suggested that the products of each myonucleus, the messenger ribonucleic acid molecules (mRNAs), are transferred directly to the sites of protein synthesis and localization. Conversely, if the multiple myonuclei function as a single nucleus (that is, if the products of different nuclei are found together in muscle fibres), then it would appear likely that there is an opportunity for gene products (mRNAs and/or proteins) to be widely distributed before they are sequestered at specific sites, or that the products remain in free solution indefinitely.

Carriers of X-linked Diseases of Muscle

The question of whether a myofibre is subdivided into territories, each under the influence of a single nucleus, is also important to our understanding of the control of muscle phenotype in human female carriers of the X-linked muscle diseases Becker and Duchenne muscular dystrophies and centrotubular myopathy. It is not known what the primary defects are in any of these diseases.

According to the Lyon hypothesis, mammalian females are genetic mosaics as a result of the random inactivation of one X-chromosome in each nucleus early in development (Lyon, 1972). Therefore, because the multinucleated condition arises by cell fusion, single myofibres of female carriers of X-linked diseases may contain some myonuclei

that express the normal X-chromosome and some myonuclei in which the active X-chromosome bears the abnormal gene. If each myonucleus maintains an exclusive territory, then such mosaic myofibres could well appear normal in the region of a normal nucleus and typically affected in the region of an abnormal nucleus. However, it has been observed that both in Duchenne muscular dystrophy (DMD) and centronuclear myopathy, there is large phenotypic variation between carriers. Muscle fibres in carriers vary in histological appearance from normal to typically affected, with many affected fibres classified as intermediate or moderately affected (Emery, 1965; Pearson, 1963; Moser and Emery, 1974; Barth, Van Wijngaarden and Bethlem, 1975). For example, muscle biopsies obtained from DMD carriers and examined by electron microscopy manifested abnormalities such as mitochondrial enlargement, myofibrillar fragmentation, and streaming of X-bands, that were qualitatively similar but less severe than the abnormalities that occur in DMD patients (Fisher, Wissinger, Gerneth and Danowski, 1972). It is not known whether individual myofibres in carriers express the same phenotype throughout their length.

There are two obvious mechanisms which could produce the intermediately affected phenotype in muscle fibres of female carriers: either the myonuclei do not control territories and, therefore, the entire mosaic myofibre has access to a mixture of normal and abnormal gene products, or the site of expression of the mutant genes may be extramuscular. In the latter case, intermediate fibres would arise when the important extramuscular tissue provides intermediate levels of a gene product essential to the development or maintenance of muscle. Therefore, if it can be shown that myonuclei do control local territories,

It will appear likely that the phenotype of muscle fibres in carriers of Duchenne muscular dystrophy and centrotubular myopathy is determined by an extramuscular influence. However, a demonstration that myonuclei cooperate in the maintenance of myofibres will not help to distinguish between a myogenic and an extramyogenic defect in the pathogenesis of these diseases.

Trophic Mechanisms

It is possible, too, that an understanding of the nuclear-cytoplasmic relationships in myofibres will help to define the mechanisms by which nerves regulate the metabolic and physiologic properties of entire muscle fibres. Many studies have addressed the question of whether specific muscle properties are regulated by the pattern of nerve-induced contractile activity or by chemical factors secreted by nerves (see review by Gutmann, 1976). If each myonucleus influences only a limited region of the myofibre, then the proposed trophic mechanisms should explain how some of the trophic effects, such as maintenance of the resting membrane potential (Albuquerque, Schuh and Kauffman, 1971) are eventually expressed throughout the length of the fibre.

DEVELOPMENT OF MUSCLE

In vertebrate embryos, mononucleated cells of mesodermal origin accumulate and proliferate at sites destined to form muscle. Overt myogenesis begins with the differentiation of bipolar, mononucleated myoblasts from the mesenchymal cells. As a group, myoblasts are mitotically active, synthesize large amounts of RNA and smaller quantities of contractile proteins and acetylcholine receptors, and are capable of fusing into multinucleated myotubes (evidence reviewed by Fischman, 1972). It is not known if only a discrete population of post-mitotic

myoblasts is capable of making contractile proteins and undergoing fusion (Holtzer, Yeoh and Rubinstein, 1977) or if these are properties of all myoblasts (Stockdale and O'Neill, 1972; Doering and Fischman, 1974). Myoblasts normally undergo cytoplasmic fusion over a short period of time in development (days 13 to 15 *in utero*, in the mouse (Pai, 1965); days 7 to 11 *in ovo* in the chick (Marchok and Herrman, 1967), with limb musculature often lagging behind trunk muscles (Fischman, 1972). The myonuclei remain distinct and are usually located in central rows along the length of myotubes.

During the period of myoblast fusion, the synthesis of contractile proteins, acetylcholine receptors, and many of the metabolic enzymes characteristic of skeletal muscle increases greatly. In addition to large accumulations of certain proteins, there are also replacements of some enzymes by other homologous proteins (isozymes) during muscle development. Well known examples are the aldolase, lactate dehydrogenase and creatine phosphokinase isozyme transitions (Cahn, 1962; Eppenberger, Eppenberger, Richterich and Aebi, 1964; Lebherz, 1965).

The mechanisms that control gene expression during myogenesis are unknown but it has been shown that many of the quantitative changes in protein synthesis can occur when myoblast fusion is blocked (Emerson and Beckner, 1974; Strohman and Moss, 1977). Therefore, cell fusion is neither an initiating nor an obligatory event in muscle differentiation. Devlin and Emerson (1978) found that the major contractile proteins are synthesized coordinately in cultured quail muscle and have suggested that a single factor regulates the expression of the genes for most of the myofibrillar proteins.

Heywood and co-workers have shown that myosin heavy chain, actin, and tropomyosin are each synthesized on a different class of polysomes in embryonic chick muscle (Heywood, Dowben and Rich, 1967; Heywood and Rich, 1968). The distribution of these different polysomes in developing muscle has not been studied but it has been noted that myosin and actin filaments usually appear together and precede the formation of Z-bands. The first myofibrils are elaborated in the peripheral region of myotubes (Fischman, 1967).

In the subsequent development of muscle cells, myofibrils continue to increase in number and in size, and come to occupy most of the cell cytoplasm. The sarcoplasmic reticulum (SR) and t-tubule networks develop around the myofibrils. The former is derived from perinuclear RER and the latter develops by invagination of the plasma membrane (Ezerman and Ishikawa, 1967). The myonuclei are eventually displaced to the periphery in most species. In cultured muscle, the basal lamina of the basement membrane first appears during cell fusion (Fischman, 1970). Both the origin and function of the basal lamina during myogenesis are poorly understood. In mature muscle, the basal lamina of the endplate appears to be a site of cholinesterase activity (McMahan, Sanes, and Marshall, 1978) and it may be involved in the differentiation of regenerating nerve terminals (Sanes, Marshall and McMahan, 1978).

Mononucleated cells termed satellite cells (Katz, 1961; Mauro, 1961) lie beneath the basement membrane but outside the plasma membrane of myofibres in immature and adult skeletal muscles of many species (Muir, 1970). It is not yet known how these mononucleated cells come to be excluded from the myotube during primary myogenesis, but it is

well established that satellite cells are potential myogenic stem cells that can proliferate and become incorporated into myofibres during postnatal growth (Allbrook, Han and Hellmuth, 1971; Moss and Leblond, 1971; Shultz, 1976) and regeneration (Konigsberg, Lipton and Konigsberg, 1975). Satellite cells in mature normal skeletal muscle appear to be rather dormant cells; the nuclear chromatin is densely clumped, the nucleolus is not prominent, there are few ribosomes, the endoplasmic reticulum and Golgi apparatus are poorly developed and the cytoplasm is scanty and contains no myofilaments (reviewed by Reznik, 1976).

The morphological differentiation of the neuromuscular junction occurs after muscle cells have developed into myofibres, although there is strong evidence that nerve contacts and functional synapses occur much earlier in myogenesis (Kelly and Zachs, 1969; Landmesser and Morris, 1975; Kullberg, Lentz and Cohen, 1977; Butler and Cosmos, 1979). The differentiation of histochemical fibre types occurs during late embryonic and early postnatal development, with the time of onset and rate of development depending on the species and muscle under consideration (Romanul, 1964; Close, 1964, 1972).

PROTEIN TURNOVER IN MUSCLE

There have been many studies of protein synthesis and breakdown in growing and mature skeletal muscle. It is clear that both RNA and protein synthesis decline in postnatal animals (reviewed by Goldspink, 1972; Burleigh, 1974). It is also known that protein turnover is sensitive to hormonal and nutritional factors (Millward, 1970; Cheek, Holt, Hill and Talbert, 1971). Rates of both synthesis and degradation are also altered during stretch-induced hypertrophy, following

denervation, and after immobilization (Goldberg, 1968; Goldspink, 1976, 1977). The mechanisms that regulate protein metabolism under many of these conditions are currently under investigation in many laboratories (see, for example, Fed. Proc. 39: 1-52).

Protein turnover rates as measured by different groups vary considerably presumably because of the variety of techniques and conditions employed. Most estimates of turnover rates are based on the rate of loss of isotope labeled proteins from the total protein pool. It is thought that isotope re-utilization frequently occurs and leads to underestimates of turnover rate (Burleigh, 1974). For the adult fowl, Gallus domesticus, it has been estimated that both myofibrillar and sarcoplasmic proteins have half-lives of 5-10 days (Laurent, Sparrow, Bates and Millward, 1978). In the adult rat, myofibrillar proteins appear to turn over with half-lives of 10-20 days and sarcoplasmic proteins with half-lives of 5-15 days (Garlick, 1969; Millward, Bates, Laurent and Lo, 1978). The half-lives of several glycolytic enzymes in adult rabbit soleus muscle were estimated to be 0.9 to 1.2 days (Dölken and Pette, 1974). In general, turnover is more rapid in red muscle (slow twitch or slow tonic fibres) than in white muscle (fast twitch fibres) (Goldberg, 1967; Lebherz, Sardo, Petell and Shackleford, 1978).

Quantitative Aspects of Protein Synthesis Along Myofibres

Few investigators have commented on the distribution of sites of protein synthesis along the length of muscle fibres. Gauthier and colleagues proposed that protein synthesis in mature, innervated myofibres occurs only in the region of the neuromuscular junction. The suggestion is based on their observation that ribosomes and polyribo-

somes are abundant only in sarcoplasm beneath the motor endplate (Padykula and Gauthier, 1970; Gauthier and Dunn, 1973). According to this scheme, proteins required elsewhere in the cell originate from this single site of synthesis. Therefore, the entire myofibre must have access to the products of each active myonucleus, whether the mRNAs are transcribed solely in the nuclei at the motor endplate, or in every myonucleus in the cell. However, Galavazi (1971) found that ribosomes are present in intermyofibrillar, subsarcolemmal and paranuclear sarcoplasm in rat myofibres, and did not report that the polyribosomes are concentrated at any particular level in the cells.

There is other indirect evidence that protein synthesis does not occur exclusively in the sarcoplasm at the neuromuscular junction. The results of several studies indicate that newly synthesized mRNA (Kabat and Rich, 1969) and contractile proteins (Morkin, 1970; Williams and Goldspink, 1974) are found along the entire length of adult muscle fibres in vivo although the site of synthesis of these products was not directly investigated.

Qualitative Aspects of Protein Synthesis Along Myofibres

It has been well established that there are a number of differences between junctional and extrajunctional regions in normal mature muscle fibres in addition to the obvious structural difference. For example, the concentration, turnover rate, and several physical and chemical properties of junctional and extrajunctional acetylcholine receptors are different in many species (Fambrough, 1979). There are also differences between the molecular forms of cholinesterase present in junctional and extrajunctional regions of muscle (Hall, 1973). In addition, the cyclic nucleotide content has been shown to differ between

endplate and nonendplate regions both in innervated and denervated muscles of the frog (Allen, Gardner, Yount and Ochs, 1978). It is not yet known how any of these differential distributions arise within a myofibre. One possibility is that the differences result when the nuclei in the junctional and extrajunctional regions direct the synthesis of different gene products and the proteins remain localized in the area of their synthesis. Conversely, all nuclei in the myofibre may direct the synthesis of the same gene products, and the assortment of these products may be induced by a neural influence.

In addition, although it has been shown that the histochemical staining properties are uniform along the length of extrafusal muscle fibres (Romanul, 1964; Farrell and Fedde, 1969), and the same proteins appear to make up the contractile machinery from end to end in myofibres, it is not known whether the uniformity results from the synthesis of identical proteins by all nuclei or the homogeneous distribution of the products of a few nuclei.

EVIDENCE FOR THE HYPOTHESIS OF NUCLEAR TERRITORIES

The hypothesis that skeletal muscle fibres are segmented into territories, each under the control of a single myonucleus, has been proposed by two groups of investigators (Ruska and Edwards, 1957; Landing et al., 1974) and has been cited by many others (e.g. Farrell and Fedde, 1969; Holtzer, 1970; Cheek et al., 1971; Reznik, 1976; Millward et al., 1978). The hypothesis was originally based on indirect morphological evidence.

Morphology

Edwards and co-workers (Edwards, Ruska, Santos and Vallejo-Freire, 1956; Ruska and Edwards, 1957) studied the ultrastructure of muscles from

insects and several vertebrate classes and concluded that each myonucleus controlled a given area of cytoplasm by way of the perinuclear endoplasmic reticulum; they observed elements that they believed to be sarcomeres at an early stage in formation in the perinuclear sarcoplasm. Evidence that tht RER forms part of the pathway for the localization of secretory, organelle-bound and membrane-bound proteins in many cell types has been referred to earlier. However, there is generally very little RER in mature muscle cells (Porter and Palade, 1957; Padykula and Gauthier, 1970; Fischman, 1972; Nihei, 1971). Andrews and Tata (1971) estimated that only 9% of muscle RNA occurs in membrane bound ribosomes. It remains questionable, therefore, that the majority of muscle proteins is synthesized on perinuclear RER in mature myofibres.

Holtzer (1970) suggested that each myonucleus comes to control its own "cytoplasmic territory" during the development of avian muscle, and Landing et al. (1974) proposed that human muscle fibres are subdivided into discrete "nuclear territories". However, these suggestions were clearly no more than speculations on the significance of the regular spacing of nuclei along the length of the myofibres examined.

There are other morphological features of the mature myofibre that are consistent with the hypothesis of nuclear territories. The sarcoplasm is densely packed with myofibrils and the small spaces between myofibrils are largely occupied by mitochondria, sarcoplasmic reticulum and the t-tubule network. In addition, there are several transverse elements within the myofibrils (the Z band, the M-line and the myosin cross-bridges). In the middle of the nineteenth century Krause introduced the idea that the Z line was a continuous transverse membrane which divided the muscle cell into small compartments

(cited in Adams, 1975). There is ultrastructural evidence that the material of the Z-band is attached to the plasma membrane or sarcoplasmic reticulum in some invertebrate muscle fibres (Franzini-Armstrong, 1979). In vertebrates, individual myofibrils are linked laterally to adjacent myofibrils at their Z-discs by desmin filaments, and each Z-plane, as the interconnected network of Z-discs is called, is linked by desmin filaments to the plasma membrane (Lazarides, 1980). It is possible that some or all of these features of myofibre structure could limit the distribution of many large cell components within muscle cells.

In spite of the potential structural obstacles in muscle cells, several observations argue that there are no rigid barriers to the longitudinal movement of cellular contents. In 1863, Kuhne reported watching a living nematode move freely up and down the inside of a muscle fibre and investigators have manipulated various inclusions (oil droplets, air bubbles) under the sarcolemma along fibres from insects and frogs (cited in Adams, 1975).

Diffusion

It has been shown that substances can diffuse along the length of mature muscle cells. Rorschach, Chang, Hazlewood and Nichols (1973) found that the diffusion coefficient of isotope-labeled water in muscle fibres of the rat gastrocnemius is $1.40 \times 10^{-5} cm^2 sec^{-1}$. Blinks, Rüdel and Taylor (1978) found that the longitudinal spread of the Ca^{++} sensitive bioluminescent protein, aequorin (M.W. approximately 30,000: Shimomura and Johnson, 1969) injected into twitch muscle fibres of the frog was consistent with a diffusion coefficient of approximately $5 \times 10^{-8} cm^2 sec^{-1}$ for the protein; the injected aequorin reached a point 1 mm on each

side of the site of injection in about 2.3 hours. For both substances, the coefficient of diffusion in muscle is small relative to the value in pure water and the reason for the slower diffusion is not clear. Rorschach et al., (1973) claim that this reduction cannot be attributed entirely to the presence of transverse membrane and filamentary elements. They suggest that the physicochemical properties of cellular water are influenced by large macromolecules in such a way that the viscosity of the cytoplasm itself is significantly increased. The general question of the physical state of water in living cells has not been answered. A minority view is that the 'soluble' proteins, water and solutes exist in an organized non-liquid state (e.g. Ling, Miller and Ochsenfeld, 1973). However, the more widely accepted view is that while some of the cellular water is bound in loose association with proteins, most of the water is free. That is, a fraction of the cytoplasm is colloidal and the remainder is an aqueous solution (e.g. Rhodin, 1974). Therefore, in muscle cells it appears likely that unbound cell components are freely diffusible in the unstructured regions of the cytoplasm and that diffusion is slow only because so little of the cytoplasm is unoccupied by structural elements.

Denervation Effects

The results of some studies of denervation effects in muscle also indicate that there is not free communication along the entire length of muscle fibres. For example, Miledi (1960) showed that when frog sartorius muscle fibres were denervated at one of their two endplates, the innervated and denervated regions of the fibre responded differently; sensitivity to acetylcholine (ACh) increased in the region around the denervated endplate but remained low in the innervated region. This

observation indicates that the gene products responsible for the development of extrajunctional sensitivity to ACh were not freely distributed throughout the length of the partially denervated fibres.

EVIDENCE AGAINST THE HYPOTHESIS OF NUCLEAR TERRITORIES

Mouse Chimaera Studies

Evidence that discrete territories do not exist in muscle fibres with respect to the maintenance of some gene products comes from studies of mouse chimaeras. Techniques have been developed for the production of single composite mice by the aggregation of 2 or more mid-cleavage eggs (Tarkowski, 1961; Mintz, 1962) or by the injection of a few blastomeres from one egg into the blastocoele of another blastocyst (Gardner, 1968). Tissues and organs from mouse chimaeras are often mosaics of cells derived from both donor embryos. Chimaeras have been used extensively in investigations of problems such as cell lineages, cell interactions, and mutant gene expression (see reviews by Mintz, 1974; McLaren, 1976; Mullen, 1977; Peterson et al., 1979).

In an attempt to determine whether the multinucleation of myofibres arises through myoblast fusion or by repeated divisions of myoblast nuclei while the cytoplasm remains undivided (endomitosis), Mintz and Baker, (1967) produced mouse chimaeras by aggregating mid-cleavage eggs from 2 inbred strains which produced different isozymes of the cytoplasmic enzyme, isocitrate dehydrogenase (Id-1). Inbred mice of different strains produce either single slow or fast electrophoretic forms of Id-1. Results of studies of Id-1 in hybrid mice indicate that the active enzyme is a dimer, the gene codes for enzyme monomers, and the 2 different types of monomers aggregate at random in heterozygous cells to produce 3 electrophoretic variants of Id-1: The slow and fast homo-

dimers and a band of intermediate mobility composed of heterodimers (Henderson, 1966). Mintz and Baker reasoned that Id-1 heterodimers would also be produced in muscle of mouse chimaeras if single fibres contained the 2 genetically different types of nuclei. They found that all 3 electrophoretic variants of Id-1 were indeed present in muscles from some chimaeras and concluded that myoblast fusion occurs during myogenesis.

Subsequently, heterodimers have been found in muscles of mouse chimaeras that were mosaic for variants of other oligomeric cytoplasmic enzymes: malic enzyme (Baker and Mintz, 1969; Peterson, 1974) and glucosephosphate isomerase (Gearhart and Mintz, 1972; Peterson et al., 1979).

These results indicate that, for each enzyme, in the mosaic muscle cells the two types of monomers or their precursors are present in a common region of cytoplasm before the assembly of enzymatically active oligomers occurs. Therefore, discrete nuclear territories cannot exist. However, the possibility remains that there are simply some regions of overlap between adjacent nuclear territories. According to the electrophoretic results published by Baker and Mintz, the proportion of heteropolymer in homogenates of whole mosaic muscles was small relative to the proportion of homopolymers. Therefore, it is possible that the heteropolymers had been generated and had remained localized only in the regions of cytoplasm between adjacent genetically different nuclei. None of the published results convey any information about the distribution of isozymes along the length of mosaic muscle cells.

Suppression of Hyperinnervation

Further indirect evidence that there may be an exchange of materials between different regions of a muscle cell is the observation that the establishment of a synapse by one nerve often induces membrane refractoriness to further innervation. For example, during the innervation of muscle fibres of the rat diaphragm, initial nerve contact at one site makes the entire fibre refractory to additional innervation (Bennett and Pettigrew, 1974). In other muscles polyneuronal, multiterminal innervation occurs during development but is eliminated at later stages (Bennett and Pettigrew, 1974; Riley, 1977). However, multiple endplates do persist in some situations, e.g. on the multiply innervated fibres of the avian anterior latissimus dorsi muscle (Purves, 1977), and on fibres of the rat soleus that are re-innervated by foreign nerves (Kuffler, Thompson, and Jansen, 1977). These observations could indicate that the factor responsible for the prevention or elimination of hyperinnervation is distributed along the entire length of some myofibres and over more limited distances in others.

AIMS OF THIS STUDY

In order to investigate the extent of the influence of myonuclei in the mammalian skeletal muscle fibre, I have extended the studies of isozymes in muscles from mouse chimaeras and specifically I have addressed two questions:

1. Are the products of different nuclei uniformly distributed along the length of mosaic muscle cells? Mosaic myofibres from mouse chimaeras are single cells in which genetically different types of myonuclei are distributed along the fibre length. Therefore, a uniform distribution of genetically determined isozymes along the length of a mosaic myofibre could indicate that the products of individual myonuclei are homogeneously distributed throughout the cell, while a non-uniform distribution would be

consistent with the hypothesis that the gene products of different nuclei largely remain localized in "nuclear territories".

2. Do enzyme monomers aggregate into oligomers in sites close to the nuclei that directed their synthesis? Although a uniform isozyme profile along the length of mosaic myofibres could indicate that the assembled enzyme is homogeneously distributed, the possibility would remain that the distribution of the monomer precursors, or the distribution of the monomers themselves, is more limited. If monomers are not uniformly distributed at all sites of oligomer assembly, there would be preferential aggregation of like monomers to form enzyme homopolymers and relatively few heteropolymers generated in mosaic muscle cells. Conversely, if gene products are widely distributed before the assembly of enzyme oligomers, then the proportion of heteropolymers will be relatively high in mosaic muscle fibre. It was possible to distinguish between these possibilities both for an enzyme that is present in the soluble fraction of muscle homogenates and for an enzyme that is localized in the mitochondrial matrix.

EXPERIMENTAL STRATEGY

Mouse chimaeras were produced by the aggregation of embryos from the SM/J and C57BL/6J inbred mouse strains. The strain composition of the chimaeras is represented by the notation SM/J ↔ C57BL/6J. This particular combination of embryos was selected because there are appropriate genetic differences between the two strains and because the results of preliminary studies had indicated that there is usually a large proportion of mosaic myofibres in skeletal muscles from SM/J ↔ C57BL/6J chimaeras.

'Soluble' Enzyme

One of the many genetic differences between SM/J and C57BL/6J mice is at the structural gene locus for glucosephosphate isomerase (GPI, D-glucose-6-phosphate ketol isomerase, E.C. 5.3.1.9). The gene locus is designated Gpi-1. Under appropriate conditions of starch gel electrophoresis, the GPI produced by SM/J mice and by mice of several other inbred strains, designated GPI-1A, migrates toward the cathode as a single band that moves more slowly than the single GPI band, GPI-1B, from mice of inbred strains such as C57BL/6J (Carter and Parr, 1967; DeLorenzo and Ruddle, 1969). Most of the activity of this glycolytic enzyme is recovered in the soluble fraction of cell homogenates, although there is some histochemical (Sigel and Pette, 1969) and biochemical (Arnold, Henning, and Pette, 1971) evidence that a fraction of the GPI is associated with myofilaments in striated muscle. In the mouse, GPI-1 activity in most cell types is high relative to many other enzymes (Carter and Yoshida, 1969).

It has been well established by in vitro hybridization (Carter and Yoshida, 1969) and genetic studies (Carter and Parr, 1967; De Lorenzo and Ruddel, 1969) that the structural gene codes for a monomer of GPI-1 while the active form of the enzyme is a dimer. In mature somatic tissues from SM/J x C57BL/6J F_1 mice, the isozyme distribution is 25% GPI-1A: 50% GPI-1AB: 25% GPI-1B (Peterson and Wong, 1978), indicating that the $Gpi\text{-}1^a$ and $Gpi\text{-}1^b$ alleles are expressed equally in each heterozygous nucleus, and that in heterozygous cells the A and B monomers are able to aggregate at random to form all 3 possible types of dimers. Although there are small differences in heat stability between the GPI-1A and GPI-LB dimers (Padua, Bullfield and Peters, 1978), it appears that the enzymatic activities of all three dimers are equal under controlled

conditions of quantitative analysis (Peterson and Wong, 1978). It would be very difficult to explain the distribution of isozyme activities observed in heterozygous tissues if the dimers have unequal activities. The specific activity has not been determined yet for any of the mouse GPI-1 isozymes.

In mosaic skeletal muscle fibres from SM/J ↔ C57BL/6J chimaeras, myonuclei derived from the SM/J mid-cleavage egg direct the synthesis of A type monomers of GPI-1, and myonuclei derived from the C57BL/6J egg encode B type monomers. The two types of nuclei become distributed along the myofibre length during development and there is no evidence that indicates that myonuclei change their positions in adult myofibres. Therefore, in order to determine whether the products of different myonuclei are uniformly distributed along the length of mosaic myofibres from SM/J ↔ C57BL/6J chimaeras, I have measured the GPI-1 isozyme ratios in serial cryostat-cut cross-sections of single mosaic myofibres and in serial segments of collagenase-isolated single mosaic myofibres. Although even the smallest samples (i.e. 20 μm thick cross-sections of single muscle fibres) contained more than one myonucleus, it is unlikely that an identical combination of nuclear types was present in all the samples from any one mosaic myofibre. Therefore, if the measured distributions of GPI-1 isozymes had been the same in every sample, it is likely that products of individual myonuclei are widely dispersed in muscle cells. Alternatively, it is to be expected that the GPI-1 isozyme distributions would have differed between sections along the length of a mosaic myofibre if gene products remain localized around the nuclei that direct their synthesis.

To investigate whether enzyme monomers are uniformly or nonuniformly distributed prior to dimer formation, the measured GPI-1 isozyme distributions were used to determine whether monomers within each sample had

aggregated at random (see Materials and Methods section for details). If the isozyme distributions reflected random monomer aggregation, then it is likely that all the sites of GPI-1 dimer formation in mosaic myofibres have access to both types of monomers, as in heterozygous cells. That is, in mosaic myofibres, monomers or their precursors must be uniformly distributed prior to dimer formation. However, if the results had indicated that there had been preferential aggregation amongst like monomers, a plausible explanation is that most of the GPI-1 monomers aggregate into dimers in territories around the nuclei that direct their synthesis.

Mitochondrial Enzyme

The other genetic difference between SM/J and C57BL/6J mice that is important to this study is at the mitochondrial malic enzyme (MOD-2, L-malate $NADP^+$ oxido-reductase, E.C. 1.1.1.40) locus. MOD-2 is encoded by nuclear DNA, synthesized on cytosolic 80S ribosomes, and eventually localized in the mitochondrial matrix (Schatz and Mason, 1974). In the mouse, enzymatically active MOD-2 is a tetramer (Bernstine, 1979). Two alleles, resulting in different electrophoretic variants, are known (Henderson, 1966). SM/J mice are homozygous for the $\underline{Mod\text{-}2^a}$ allele and produce a single band, MOD-2A, composed of AAAA tetramers. All other inbred strains, including C57BL/6J are homozygous for $\underline{Mod\text{-}2^b}$ and produce only the BBBB tetramers. The Mod-2 isozyme distribution in $\underline{Mod\text{-}2^a}/\underline{Mod\text{-}2^b}$ heterozygotes has not been clearly resolved, but the pattern appears to be composed of 5 bands: the 2 parental forms, AAAA and BBBB, and the three heteropolymer forms, AAAB, AABB and ABBB (Shows, Chapman and Ruddle, 1970).

MOD-2 activity cannot be detected in homogenates of single muscle cells with techniques currently available. It was my aim simply to determine

whether or not MOD-2 heteropolymers are present in the mitochondria of mosaic muscle fibres from $\underline{MOD\text{-}2^a}/\underline{Mod\text{-}2^a} \leftrightarrow \underline{Mod\text{-}2^b}/\underline{Mod\text{-}2^b}$ chimaeras. If monomers or monomer precursors of MOD-2 are widely distributed before oligomer assembly and/or incorporation into mitochondria, then heteropolymers would have been recovered from mosaic muscle fibres. Alternatively, if the mechanisms that exist for the localization of nuclear-coded mitochondrial proteins link each myonucleus directly and exclusively with the mitochondria in its vicinity, then each mitochondrion in these mosaic muscles would have contained only one or the other form of MOD-2 tetramer and MOD-2 heteropolymers would not have been observed.

New Techniques Used in the Study.

Relatively simple techniques were available for the separation and detection of isozymes of GPI-1 (Carter and Parr,1967; De Lorenzo and Ruddle, 1969) but the methods were not sensitive enough to measure the isozyme distribution in cross-sections of single muscle cells. During the course of the study, a procedure was developed for the accurate quantitative analysis of very small tissue samples (Peterson, Frair, and Wong, 1978) including sections of single myofibres. Techniques were also developed for the recovery and handling of serial cryostat-cut sections and enzymatically dissociated segments of individual muscle fibres.

Existing techniques for the separation and visualization of MOD-2 isozymes on starch gels were modified to accommodate small volume samples and to improve the resolution and sensitivity of the analysis.

Collaboration

The preparation of mitochondrial extracts from whole cells and from isolated mitochondria, and the staining of gels for MOD-2 activity were done in collaboration with Drs. Strasberg and Freeman of the Department of Biochemistry at McMaster University.

SUMMARY OF RESULTS

The results of my investigations indicate that skeletal muscle cells are not subdivided into nuclear territories; rather, multiple myonuclei contribute gene products to each region of the myofibre.

MATERIALS AND METHODS

MATERIALS AND METHODS

ANIMALS

All mice were housed in the Animal Facilities at McMaster University Health Sciences Centre. They were fed a standard diet with 11% fat content (Purina Mouse Chow), and kept on a 14-hour light cycle (light 0500-1900 hours). Mice used for the production of chimaeras were bred at McMaster from stocks obtained from the Jackson Laboratory, Bar Harbour, Maine.

CHIMAERA PRODUCTION

The procedure for the production of mouse chimaeras was essentially that described by Mullen and Whitten (1971), modified after Tarkowski (1961) and Mintz (1962).

Production Schedule

Day -1,	1600-1800 hr.	Pair strain A ♀ with A ♂ Pair strain B ♀ with B ♂
Day 0,	0800-1000 hr.	Observe and record copulation plugs in A and B ♀'s.
	1600-1800 hr.	Pair recipient ♀ with vasectomized ♂
Day 1,	0800-1000 hr.	Observe and record copulation plugs in recipient ♀'s.
Day 2,	1200-1500 hr.	Collect and aggregate A and B strain eggs.
Day 3,	1500-1700 hr.	Transplant blastocysts into pseudopregnant recipient ♀'s.
Day 20,		Observe and record litters born to recipient ♀'s or deliver surgically and present to foster ♀.

Timed Matings

Donor and recipient females were mated during proestrus and judged by the external appearance of the vagina.

Collection of Eggs

Fertilized eggs were collected from females of the two donor strains on Day 2. A donor was killed by cervical dislocation and the oviduct and uterine horn on each side was removed and placed on a watch glass (4cm square with a 3 cm diameter cavity). For each side, the uterotubal junction was held with curved forceps and the oviduct was flushed using a 3 ml hypodermic syringe fitted with a short bevel 30 gauge needle (Popper and Sons, Inc.) filled with warm (37°C) Brinster's culture medium (BMOC-3), Grand Island Biological Company). Mid-cleavage eggs at the 8-celled stage were collected in a glass micropipette, washed in warm Brinster's medium, transferred to a solution of pronase (B grade, Calbiochem, 5 mg/ml in Brinster's medium) for 2-5 minutes to remove the zonae pellucidae, and then washed three times in fresh warm Brinster's medium.

Culture of Morulae

Approximately 1 hr. before eggs were collected, paraffin oil (white, Seybolt Viscosity 125, Fisher Scientific Company) was equilibrated with Hank's balanced salt solution (Hank's BSS, Grand Island Biological Company) and an atmosphere of 5% CO_2/95% air; the warmed gas was bubbled through the mixture of oil and Hank's BSS for 10-15 minutes. The oil was separated from the Hank's BSS by centrifugation and placed in 10-12 ml. lots in Falcon plastic tissue culture dishes (60 x 15 mm). Fifteen to twenty-five separate drops of Brinster's

medium (3-5 mm in diameter) were introduced under the oil and adhered to the plastic surface of the culture dish. The culture dish was covered and placed in a stainless steel culture jar (Torbal, Fisher Scientific Company) that was gassed with 5% CO_2 in air, sealed and placed in an incubator at 37°C until the eggs were collected.

One zona-free morula of each donor strain was placed in a culture drop and the pair pushed together. The culture dishes were covered and returned to the culture jar and the jar was gassed, sealed and placed in the incubator. Two to three hours later the culture dishes were observed once more and any separated pairs of embryos were re-aggregated.

Transfer of Blastocysts

After twenty-four hours in culture, each pair of aggregated morulae usually developed into a single, composite blastocyst. The giant blastocysts were collected, washed once in warm Brinster's medium and transplanted into pseudopregnant recipient mice. The transplant procedure is described below.

Recipient mice were anaesthetized with sodium pentobarbital (Nembutal, 85 mg/kg I.P.). A 10-15 mm long incision was made in the skin along the dorsal midline and the skin pulled back to expose the musculature overlying the uterus, oviduct and ovary on the right side of the animal. A small incision (approximately 3 mm long) was made in the musculature and the cranial portion of the right uterine horn was gently pulled through the incision. A thread was passed through the mesometrium and was used to hold the uterus while the blastocysts were injected through a hole made by puncturing the uterus with a 27 gauge hypodermic needle. Care was taken to inject only a minimal volume of medium and no air into the uterus. Up to 10 blastocysts were transplanted to a single recipient. After the transfer of blastocysts, the uterus was gently

pushed back inside the body cavity and the incision in the body wall was closed with a suture. The skin incision was closed with stainless steel wound clips. After surgery the recipient mice were caged individually.

Delivery and Care of Chimaeras

Recipient female mice that had not delivered litters by midafternoon of the seventeenth day after the transfer of blastocysts were killed and any fetuses were removed from the uterus. Full-term animals delivered in this manner usually began breathing on their own and were caged with a foster female mouse. Recipient females that had borne litters naturally were allowed to nurse their litters.

ANALYSIS OF GPI-1 ISOZYMES

Preparation of Samples

1. Frozen Sections

Muscles were removed from freshly killed or anaesthetized mice (Nembutal, 85mg/kg), mounted in minced liver on cork discs, and immersed for 15 seconds in isopentane (Practical Grade, J.T. Baker Chemical Co.) cooled with liquid nitrogen. The muscles were stored in sealed containers at -80°C until sectioned. Frozen sections were cut at a thickness of 20, 30 or 40 µm in an American Optical Cryo-Cut model cryostat at -22°C. Whole muscle sections were either homogenized for the determination of total GPI-1, or prepared for the dissection of single muscle fibres.

For the determination of total GPI-1, a sample of one or several frozen sections of the whole muscle was homogenized for 60 sec in 5 µl of cold (4°C) homogenization buffer in an ice-cold ground glass homogenizing tube. Each tube was fitted with a matched ground glass pestle for complete

disruption of the sample. The homogenization buffer was 2.5 mg/ml bovine serum albumin (Fraction V, Sigma) in GPI-1 gel buffer. The pestles were removed and the homogenates were centrifuged at 27,000g for 30 min. at 4°C. Approximately 1 µl aliquots of the supernatant were electrophoresed.

For the isolation of single muscle fibres, a frozen section was melted on to a circular glass coverslip (22 mm diameter) which had been coated with a 50% w/v solution of polyvinylpyrolidone (PVP, 10,000 MW, Sigma). The PVP acted as a gentle fixative; it preserved the morphology of the cut muscle fibres without significantly reducing the activity of GPI (Burstone, 1957). After the section had been picked up, a perspex O-ring was sealed with silicone grease (Dow Corning High Vacuum Grease) around the margin of the coverslip. The section was covered with silicone oil (Dow Corning 200-fluid) to a depth equal to the thickness of the O-ring (1.5 mm). The silicone oil prevented rapid dehydration of the section.

The prepared section was examined under differential interference contrast conditions at 80X magnification using a Reichert inverted microscope. Selected areas of the section were photographed with a Polaroid camera and numbers assigned to individual muscle fibres on the prints. Identified fibres were dissected from the section with a tungsten needle and homogenized in approximately 1 µl of homogenization buffer. With the aid of the photographs, a particular fibre could be identified and dissected from other cross sections of the muscle (Figure 2).

A microsystem was designed for the rapid homogenization and essentially complete recovery of these small volume samples. Homogenization tubes of 1-3 µl capacity were made from heat-sealed glass capillary tubes. A high speed electric rotary motor (Dremel Tool Corporation) was adapted

Figure 2

Series of photographs taken during the isolation of a single muscle fibre from a 20μm thick cross-section of a mouse anterior tibialis muscle. The diameters of muscle fibres in the mouse range from approximately 20 to 80 μm.

to drive a small platinum wire homogenizing loop (approximately 0.5 mm diameter) mounted in a 26 gauge hypodermic needle. Samples were homogenized at 45,000 r.p.m. for 5 seconds. The loop was washed in a tube containing homogenization buffer alone between samples. Occasionally the washing solution was taken for GPI-1 analysis to determine whether samples could be contaminated during homogenization. GPI-1 activity was never detected in the loop wash.

Once homogenized, the single muscle fibre samples were centrifuged for 30 seconds in a microhaematocrit centrifuge (Adams Readacrit). The entire volume of supernatant of each sample was electrophoresed.

2. Cells Isolated with Collagenase

Muscles or, in the case of fetal and newborn animals, the entire skinned hindlimbs were removed from anaesthetized or freshly killed mice. The tissues were immediately placed in 5 ml of Ca^{++}-free, Mg^{++}-free Hank's balanced salt solution (CMF Hank's, Grand Island Biological Company) at 37°C for 15-20 minutes, and then transferred to a 25 ml Erlenmyer flask containing 5 ml of a 0.3% collagenase solution (Sigma Type I, in CMF Hank's) at 37°C. The flask was covered and placed in a shaking water bath (37°C, 60 shakes/min) for 15 minutes. The enzymatic digestion was stopped by removing the tissue to a Falcon plastic petri dish (60x15 mm) containing 15 ml of ice-cold CMF Hank's. The muscles were gently agitated and the dish was placed on the stage of a Zeiss dissecting microscope. Fibres released by the enzymatic and mechanical disruption were examined at 100X magnification to confirm that they were single fibres. Once identified, single muscle fibres were usually picked up on a microdissecting needle and transferred to homogenization tubes containing approximately 1 µl of buffer. Longer segments of single fibres (over 2 mm) were pipetted on to a glass

microscope slide and cut into two or more pieces. Each piece was then treated as an individual sample. Samples containing segments of single muscle fibres were homogenized and centrifuged by the method described for samples containing dissected sections of single fibres.

3. Red Blood Cell Lysates

Fresh blood was collected in a heparinized glass capillary tube (0.1 ml volume) and transferred to a 1 ml plastic centrifuge tube (Eppendorf) containing 0.5 ml of GPI-1 homogenization buffer. Each sample was frozen and thawed three times to completely disrupt the cells and, finally, centrifuged in an Eppendorf desk-top centrifuge for 1 min. Aliquots of the supernatant were electrophoresed.

Electrophoresis

The microstarch gel technique of Tsuyuki, Roberts, Kerr, and Ronald, (1966) was adapted for the horizontal high voltage electrophoresis of GPI-1. The conditions of electrophoresis were:

1. Buffer System

The buffer system was a modification of that reported by Erickson et al. (1974). The bridge buffer was 100mM citric acid (anhydrous, J.T. Baker Chemical Co.) adjusted to pH 7.2 with Trizma base (Tris (hydroxymethyl) aminomethane, reagent grade, Sigma Chemical Co.). The gel buffer was 7 ml of bridge buffer diluted to a final volume of 250 ml with distilled water (i.e. 2.8mM citric acid).

2. Starch Gel

A 15.2% starch gel was made by adding 250 ml of gel buffer to 38 g of hydrolyzed potato starch (Lot 307, Electrostarch Company, Madison, Wisconsin). The gel was cooked to the supplier's specifications and allowed to set at room temperature in the gel mold (17.5 x 3.5 x 4 cm deep) for

2 hrs before it was cut. Gel strips of 1.5 mm thickness were cut and supported between Mylar film (Johnson Industrial Plastics, Toronto). Sample wells were cut in each strip and the samples were applied with fine glass micropipettes.

3. Running Conditions

Electrophoresis was carried out at 4°C . Power was supplied by an Ortec 4100 pulsed constant power supply (Oak Ridge Technical) at 0.1 μF discharge capacitance and 500 pulses/sec. The gels were run for 1350 Volt hours at a voltage gradient of 30V per cm.

4. Starch Lot 303

Early in the study, Electrostarch of Lot 303 was used. Best electrophoretic results were obtained with gels from Lot 303 starch when the bridge buffer was 47mM citric acid, the gel buffer was 1.32mM citric acid, the gel was 16.8% starch, and the gels were run for 1000 Volt hours at a voltage gradient of 33V per cm.

Enzyme Localization

GPI activity was detected by a modification of the histochemical staining procedure reported by Carter and Parr (1967). The sequence of reactions which leads to the production of the visible stain, formazan dye, is:

$$\text{Fructose-6-phosphate} \xrightarrow{\text{glucosephosphate isomerase}} \text{glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{6-phosphogluconate} + NADPH$$

$$NADPH + \text{phenazine methosulphate (PMS)} \rightarrow NADP^+ + PMSH_2$$

$$PMSH_2 + \text{nitroblue tetrazolium (NBT)} \rightarrow PMS + \text{reduced NBT (formazan)}$$

The sensitivity of the staining technique was increased significantly by the use of a filter overlay system, apparently because the nitrocellulose filter matrix traps virtually all the formazan produced. The filters are

also more conveniently stored and more easily cleared for densitometric analysis than are conventional starch electrophoretograms.

1. Filter Overlay Technique

The filter overlays were assembled by covering a rubber O-ring (45 mm outer diameter, 40 mm inner diameter) on one side with silicone grease (Dow Corning High Vacuum Grease) and pressing the ring firmly on to the less polished surface of a numbered nitrocellulose filter disc (0.45 µm pore size, 47mm diameter, Sartorius Membrafilter). Following electrophoresis, the section of each gel strip containing the origin and the zone of GPI-1 migration (cathodal) was cut out and placed on a glass microscope slide (75x50 mm). A filter overlay was placed over the GPI-1 zone. When the filter was uniformly wet and adsorbed to the gel, the 6.5 ml staining well formed by the filter and O-ring was filled with staining solution. The assembly was covered with a second glass slide and incubated in the dark at 37°C.

2. Staining Solution

The staining solution was 20mM citrate buffer, adjusted to pH 8.0 with Trizma base, and containing 1 unit/ml. glucose-6-phosphate dehydrogenase (Sigma Type XII from Torula yeast), 4.65 mM D-fructose-6-phosphate (disodium salt), 2.5×10^{-4}M. β-$NADP^+$ (or beta-$NADP^+$) (sodium salt), 1.1×10^{-4}M. phenazine methosulphate, 2.3×10^{-4}M. nitroblue tetrazolium (Sigma Grade III), and 30mM $MgCl_2$. All stain components were obtained from Sigma Chemical Company, except for citric acid and $MgCl_2$, which were obtained from the J.T. Baker Chemical Company.

3. Fixing and Storage of Electrophoretograms

Stained filters were rinsed with distilled water, the O-rings were removed and the filters were lifted from the starch gel surface. Each filter

was trimmed to remove all traces of silicone grease, and fixed for at least 1 hr in a solution of water, methanol, and glacial acetic acid (5:5:1 by volume). Fixed filters were dried overnight at 37°C. Dried filters were photographed and either cleared immediately for densitometry or stored in the dark.

Densitometric Analysis of Electrophoretograms

The relative amount of each GPI-1 isozyme in a sample was assumed to be equal to the relative staining intensity of each band on the electrophoretogram. The assumption appears to be valid over the range of GPI-1 activity of the samples analyzed in this study (Klebe, 1975; Peterson and Wong, 1978).

The stained, fixed, dried nitrocellulose filters were cleared for densitometry by immersion for a few seconds in cedarwood oil (J.T. Baker Chemical Co.). The cleared filters were sandwiched between two glass slides (75x50) mm) and the zone of GPI activity was scanned in a Clifford densitometer (Model 345, Fisher Scientific). The Clifford Model 345 densitometer is equipped with an electronic integration system that automatically calculates the percentage of the total density that occurs in each peak. Each sample was scanned three times and the results averaged to determine the relative activity in each isozyme band.

Calculation of Expected GPI-1 Distributions

For each observed distribution of GPI-1 isozymes, the theoretical distribution that would have resulted from random association of the monomers was calculated. In the general case, if the observed proportion is xAA:y AB:zBB, then the ratio of A:B monomers present in the sample is $(x + (y/2)):((y/2) + z)$. The proportions of AA, AB and BB dimers that would

have resulted from randomly selecting monomers two at a time from the population are given by the coefficients of the binomial expansion $\{(x + (y/2)) + ((y/2) + z)\}^2$. For example, the measured isozyme distribution in a single muscle fibre from a $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^b}$ mouse was 21.5% AA: 56.0% AB: 22.5% BB. Therefore, the A:B ratio in the sample was 49.5:50.5 and the expected proportions were 24.5% AA: 50.0% AB: 25.5% BB.

Control Samples and Statistical Tests

1. Differences Between Samples

One of the major questions asked in this study was whether there were differences in GPI-1 isozyme distributions between samples from mouse chimaeras. However, it was clear that repeated quantitative analyses of identical samples did not produce identical results; the measured isozyme distributions varied in spite of efforts to carry out sample preparation, electrophoresis, staining and densitometry under identical conditions. It was expected that some differences between experimental samples would also arise because of errors in the quantitative analysis system.

To obtain appropriate estimates of the differences in isozyme distribution that could be expected to arise between chimaera samples as a result of experimental error, the isozyme profiles in serial cross-sections of whole muscles, and of single muscle fibres, from $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^b}$ mice were measured. Myonuclei along the length of skeletal muscle fibres from $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^b}$ mice are genetically identical with respect to the synthesis of GPI-1 and, therefore, the GPI-1 isozyme distribution is identical throughout these myofibres. Thus, the apparent differences between samples from $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^b}$ mice were due to experimental error.

In order to determine whether the differences in isozyme distribution

between chimaera samples were greater than the differences expected to arise due to experimental error, the differences between samples from $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^b}$ mice (control) were compared to the differences between samples from chimaeras (experimental) by the Mann-Whitney test for a difference between 2 means (Freund, 1967).

2. Aggregation of Monomers

A second major question was whether the monomers of GPI-1 had aggregated into dimers at random in experimental samples. In order to determine whether aggregation had been random or nonrandom, the difference was calculated between the proportion of GPI-1 activity measured in the heteropolymer band and the proportion of heteropolymer predicted on the basis of random aggregation. Examination of the results for samples from $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^b}$ mice, in which the random aggregation of monomers results in an isozyme distribution of 25% GPI-1A: 50% GPI-1AB: 25% GPI-1B (DeLorenzo and Ruddle, 1969; Peterson and Wong, 1978), revealed that, in any single sample, the measured proportion of GPI-1AB was seldom equal to the predicted value. Both the measured value and the predicted value (since it was calculated from the measured isozyme distribution) were subject to experimental error. Therefore, in order to test whether there were real differences between the measured and predicted proportions of GPI-1AB in chimaera samples, the experimental (measured minus predicted) values were compared to the (measured minus predicted) values for a group of samples from control $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^b}$ mice by the Mann-Whitney test for a difference between 2 means (Freund, 1967).

ANALYSIS OF MOD-2 ISOZYMES

Preparation of Samples

Whole cell extracts were obtained from frozen cross sections of skeletal and cardiac muscles. The procedure for the dissection, freezing and sectioning of tissues was described above. Approximately 5-10 mg of tissue (15-30 sections of 20 µm thickness) were homogenized by hand in 5 µl of extraction buffer (10mM phosphate buffer, pH 7.4, containing 5mM L-malate, $2x10^{-5}$M beta-mercaptoethanol, 0.1% Triton-X 100) for 60 sec in an ice-cold ground glass homogenizer. Homogenates were then sonicated for 5 min (Cole Parmer Model 8845, ice-cold) and centrifuged at 27,000 g for 30 min at 4°C. Aliquots of the supernatants were electrophoresed.

Mitochondria were isolated from 100-200 mg of fresh unfrozen muscle by the procedure of Spiro, Moore, Prineas, Strasberg and Rapin (1970) and disrupted by homogenization and sonication as above.

Electrophoresis

The horizontal electrophoresis of MOD-2 isozymes was carried out using the microstarch gel system (Tsuyuki et al, 1966). The procedure for MOD-2 electrophoresis differed from that described for GPI-1 only in the following details:

1. Buffer System

The bridge buffer was 47mM citric acid (anhydrous, J.T. Baker Chemical Co.) adjusted to pH 8.6 with Trizma base (powder, reagent grade, Sigma Chemical Co.). The gel buffer was 12.5 ml of bridge buffer diluted to a final volume of 250 ml with distilled water.

2. Starch Gel

A 14% starch gel was prepared. Starch from Electrostarch Lot 303 was always used.

3. Running Conditions

Electrophoresis was conducted for 1250 Volt hours at a voltage gradient of 15V/cm.

Enzyme Localization

MOD-2 catalyzes the oxidation of L-malate and the reduction of $NADP^+$. Therefore, MOD-2 activity on the starch gels was detected as areas of NADPH fluorescence elicited by ultraviolet illumination of gels which had been incubated in the presence of L-malate and NADP+ (Strasberg, Davidson, Wallace and Freeman, 1974). This method was used since the routine MOD-2 staining reaction involving the reduction of nitroblue tetrazolium dye (Henderson, 1966) was not sufficiently sensitive.

Following electrophoresis, the entire gel strips were removed from the bridge tank and were incubated at 37°C in the staining solution. The staining solution was 40mM triethanolamine adjusted to pH 7.5 with HCl, and containing 4mM $MnCl_2$, 5 mM L-malate (monosodium salt), and 2.6×10^{-4} M β-$NADP^+$ (sodium salt). All chemicals were obtained from Sigma Chemical Company.

In most samples, fluorescence was detected after a 15 min incubation. The enzyme activity in each sample was between 1.0 and 2.0×10^{-5} units (1 unit is equivalent to the activity which transforms 1 µmol substrate per minute at 25°C). To provide a permanent record of the results, the electrophoretograms were photographed immediately after the incubation.

RESULTS

RESULTS

ANALYSIS OF GPI-1 ISOZYMES

Electrophoretic and Staining System

1. Resolution of Isozymes

The genetically determined variants of GPI-1 were clearly resolved in the starch gel electrophoretic system. The relative migrations of the GPI-1A and GPI-1B isozymes, as well as the distribution of isozymes in a $Gpi\text{-}1^a/Gpi\text{-}1^b$ heterozygous mouse are shown in Figure 3. The densitometric profile of the electrophoretogram of the heterozygote sample is also shown in Figure 3.

2. Origin of GPI-1

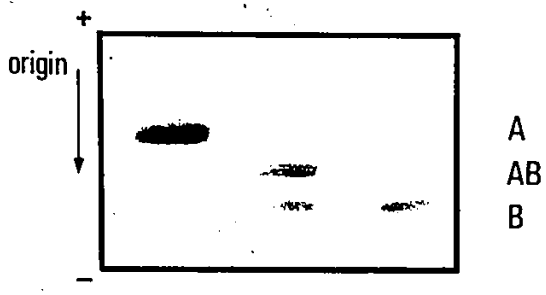
In this study GPI-1 heterodimers were recovered from only two sources: somatic cells of $Gpi\text{-}1^a/Gpi\text{-}1^b$ heterozygous mice and skeletal muscle cells of $Gpi\text{-}1^a/Gpi\text{-}1^a \leftrightarrow Gpi\text{-}1^b/Gpi\text{-}1^b$ mouse chimaeras. All these cells contained the genes for both types of GPI-1 monomers. GPI-1AB was not generated by the dissociation of homodimers and reaggregation of monomers to form heterodimers during sample preparation or electrophoresis as only the two homodimer bands were present in mixed samples containing tissues from homozygous $Gpi\text{-}1^a/Gpi\text{-}1^a$ and $Gpi\text{-}1^b/Gpi\text{-}1^b$ mice. In addition, there was no evidence that heterodimers could arise from the exchange of GPI-1 monomers between mononucleated cells *in vivo*. Heterodimers were not recovered from cardiac muscle, liver, or blood of $Gpi\text{-}1^a/Gpi\text{-}1^a \leftrightarrow Gpi\text{-}1^b/Gpi\text{-}1^b$ mouse chimaeras. These results are in agreement with previous reports that there is no intercellular exchange of monomers of either GPI-1 or isocitrate dehydrogenase (Baker and Mintz, 1969) in

Figure 3

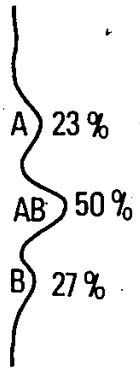
a) Electrophoretograms of the genetically determined variants of GPI-1 (photograph of filter overlay). The origins, with 5 mm slots, are located at the upper part of the photograph. Under the conditions used in this study, GPI-1 migrates toward the cathode. $\underline{Gpi\text{-}1}^{a}/\underline{Gpi\text{-}1}^{a}$ mice produce a single band, designated GPI-1A, that migrates with a lower electrophoretic mobility than the single band produced in $\underline{Gpi\text{-}1}^{b}/\underline{Gpi\text{-}1}^{b}$ mice and designated GPI-1B. Both the slow and fast enzymes are composed of homodimers; GPI-1A is composed of two identical A monomers and GPI-1B is composed of two identical B monomers. In mature somatic cells of $\underline{Gpi\text{-}1}^{a}/\underline{Gpi\text{-}1}^{b}$ mice, a band of intermediate electrophoretic mobility, composed of AB heterodimers, is produced in addition to the two types of homodimers.

b) Densitometric profile of the GPI-1 isozyme distribution in the heterozygote sample shown above. The filter was cleared and the three bands were scanned in a Clifford model 345 densitometer. The percentage of the total stain present in each of the three bands is indicated.

a)



b)



appropriate mouse chimaeras. On the basis of these observations, it was assumed that the measured GPI-1 isozyme distributions are valid estimates of the intracellular distributions, and that the nuclei within each muscle cell are the only nuclei that are responsible for the synthesis of the GPI-1 present in that cell.

3. Sensitivity of the Overlay Staining Technique

The staining procedure allowed the resolution of GPI-1 isozymes from very small tissue samples, (e.g.) a cryostat cross-section of a single muscle fibre, a few hundred erythrocytes, or a single mouse oocyte (wet weight of these samples are approximately 5, 50, and 180 ng respectively). Examples of electrophoretic results from these samples are shown in Figure 4.

4. Accuracy of the Quantitative Analysis

To assess the accuracy of the quantitative analysis, samples of somatic tissues from mature $Gpi\text{-}1^a/Gpi\text{-}1^b$ mice, in which the isozyme distribution was known to be 25% GPI-1A: 50% GPI-1AB: 25% GPI-1B (Peterson and Wong, 1978), were analyzed at various times over the course of the study. Based on the results for 39 samples run on 14 gels, the overall mean and standard deviation values for the proportion of GPI-1 activity in each isozyme band were 24.1 ± 4.2% GPI-1A: 49.2 ± 7.0% GPI-1AB: 26.7 ± 4.2% GPI-1B. That is, when identical samples were analyzed on a large number of gels, the mean of the measured isozyme distributions was accurate. However, as indicated by the standard deviation values, there was considerable variability from sample to sample in the measurements of isozyme distributions, even though the samples, in fact, contained identical isozyme distributions. The variability was due to experimental error in the quantitative analysis system.

Figure 4

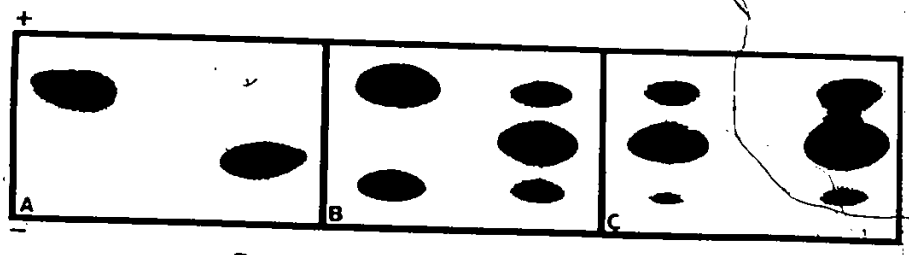
Detection of GPI-1 from small samples.

A) On the left, an unfertilized ovum from a $\underline{Gpi\text{-}1}^{a}/\underline{Gpi\text{-}1}^{a}$ mouse and, on the right, an unfertilized ovum from a $\underline{Gpi\text{-}1}^{b}/\underline{Gpi\text{-}1}^{b}$ mouse. Staining time 12 hr.

B) Mouse red blood cell lysates. On the left, a mixture of approximately 13,000 red blood cells each from a $\underline{Gpi\text{-}1}^{a}/\underline{Gpi\text{-}1}^{a}$ mouse and a $\underline{Gpi\text{-}1}^{b}/\underline{Gpi\text{-}1}^{b}$ mouse. On the right, approximately 26,000 red blood cells from a $\underline{Gpi\text{-}1}^{a}/\underline{Gpi\text{-}1}^{b}$ mouse. Staining time 2 hr 15 min.

C) Each sample was a 20 µm thick cross-section of a single muscle fibre from the anterior tibialis muscle of a $\underline{Gpi\text{-}1}^{a}/\underline{Gpi\text{-}1}^{b}$ mouse.

While the isozyme distributions as measured in samples from heterozygous mice were seldom exactly equal to the expected 25% GPI-1A: 50% GPI-1AB: 25% GPI-1B distribution, the actual deviation from the expected distribution is exaggerated in some photographs of electrophoretograms. In these photographs, the GPI-1B band in heterozygous samples appears to contain much less than 25% of the total GPI-1 activity. The relative proportion of GPI-1B measured in the samples was between 19 and 22 %.


+
A
B
C
−

5. Control Samples

Differences between samples

In order to determine whether or not there were real differences in GPI-1 isozyme profiles between samples from mouse chimaeras, it was necessary to know what apparent differences between samples could be expected to arise due to experimental error. These estimates were obtained from analyses of GPI-1 isozyme distributions in samples from $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^b}$ mice.

The apparent differences in GPI-1 isozyme profiles between adjacent cross-sections of single skeletal muscle fibres and between adjacent whole muscle cross-sections from $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^b}$ mice are summarized in frequency histograms in Figures 5, 6, and 7, (original data in Appendix 1). It is apparent that small differences were measured more often than large differences. However, all these differences were due to error in the quantitative analysis; it is known that the GPI-1 isozyme profile is uniform throughout the somatic tissues of heterozygous mice. Therefore, the values for the apparent differences between sections from heterozygous mice comprised groups of control data that could be compared to the differences observed in appropriate groups of experimental samples using nonparametric statistical tests (see Materials and Methods).

Monomer aggregation

To determine whether, within samples from house chimaeras, monomers of GPI-1 had aggregated at random, the measured proportions of GPI-1AB were compared to the proportions predicted to arise if all the monomers had randomly aggregated. If there had been preferential aggregation among like monomers, then the measured proportions of GPI-1AB would have been less than the predicted amounts.

Figure 5

Apparent differences in GPI-1 isozyme distribution between identical control samples from *Gpi-1*^{a}/*Gpi-1*^{b} mice. The differences in the measured percentage of each GPI-1 band between adjacent cross-sections of whole muscles (broken lines) and between adjacent cross-sections of single muscle fibres (solid lines) from heterozygous mice are summarized in frequency histograms. Each of the frequency distributions approximates the right half of a normal distribution; that is, small differences between adjacent sections occurred more frequently than large differences. The apparent differences are due to experimental error. Section thickness 20 µm.

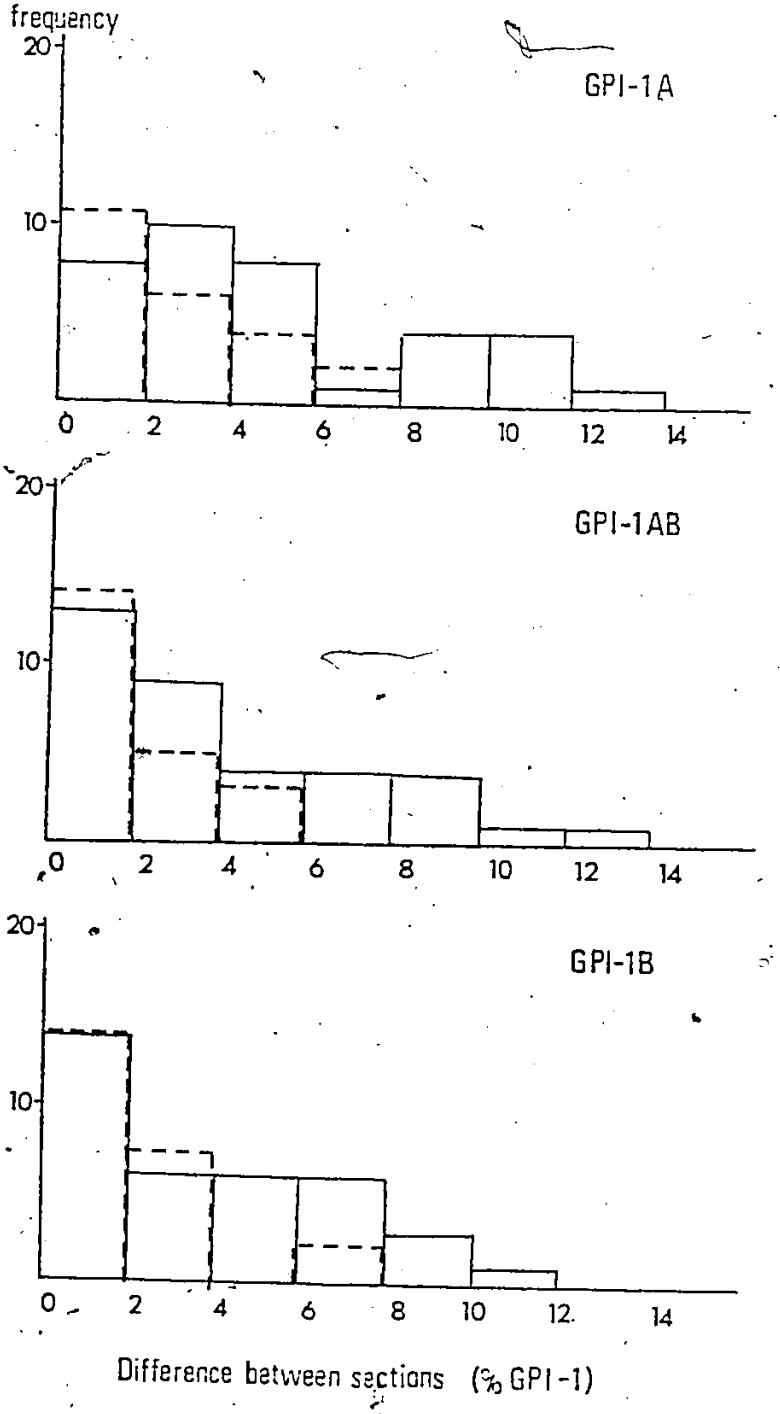

frequency
20
10
GPI-1A
0
2
4
6
8
10
12
14
20
10
GPI-1AB
0
2
4
6
8
10
12
14
20
10
GPI-1B
0
2
4
6
8
10
12
14
Difference between sections (% GPI-1)

Figure 6

Frequency distributions of the apparent differences in the measured percentage of each GPI-1 isozyme band between adjacent whole muscle cross-sections (broken lines) and between adjacent single muscle fibre cross-sections (solid lines) from Gpi-1^a/Gpi-1^b mice. Section thickness 30 µm.

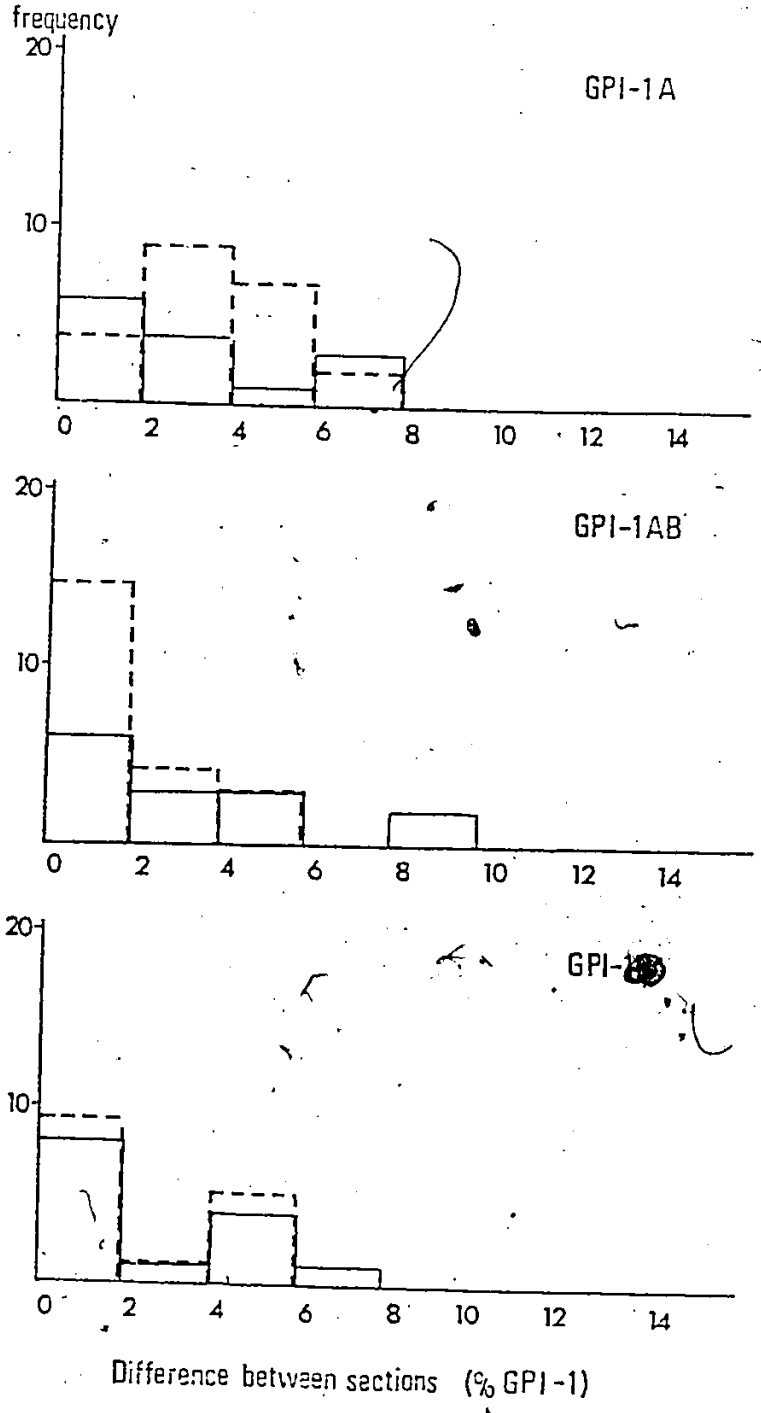
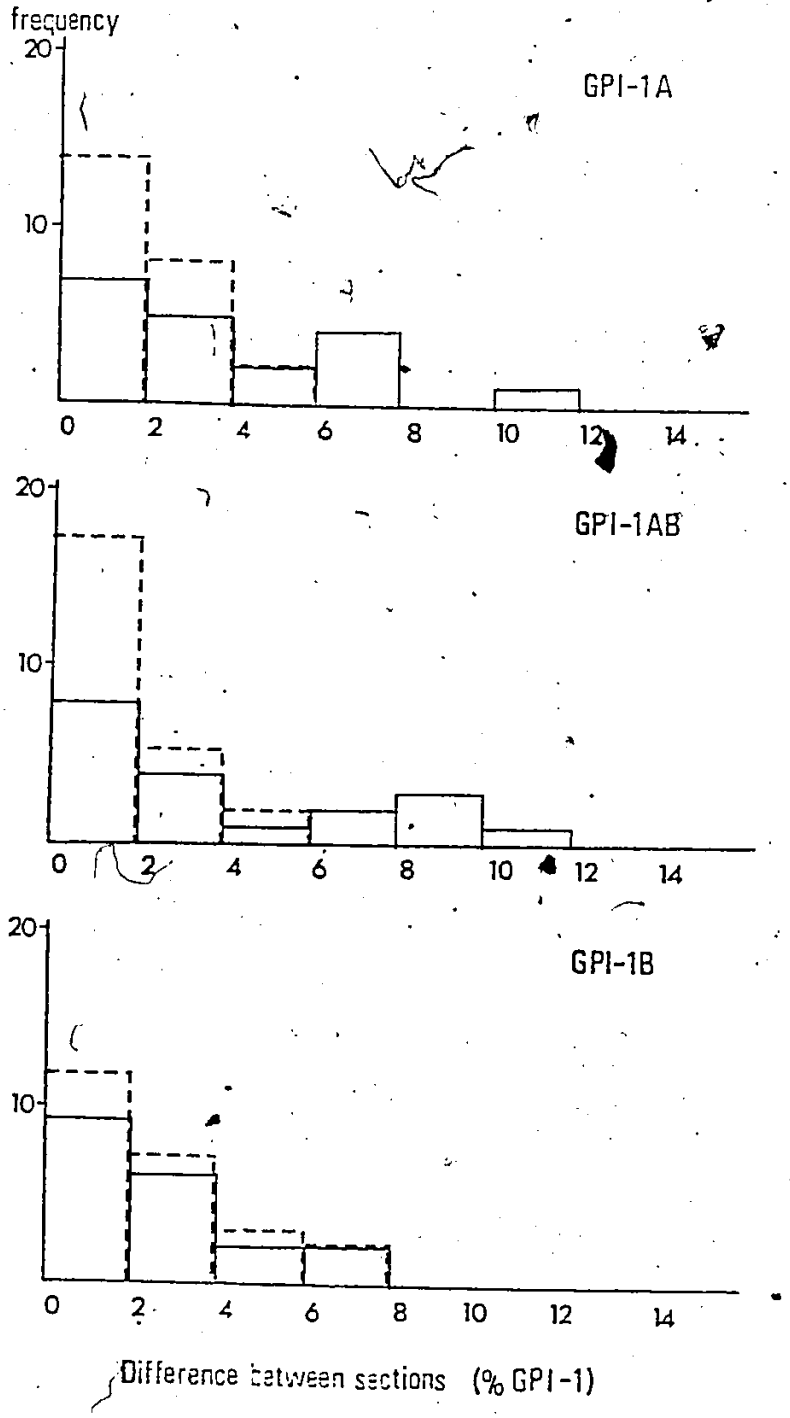

frequency
20
10
GPI-1A
0 2 4 6 8 10 12 14
20
10
GPI-1AB
0 2 4 6 8 10 12 14
20
10
GPI-1B
0 2 4 6 8 10 12 14
Difference between sections (% GPI-1)

Figure 7

Frequency distributions of the apparent differences in the measured percentage of each GPI-1 isozyme band between adjacent whole muscle cross-sections (broken lines) and between adjacent single muscle fibre cross-sections (solid lines) from Gpi-1^{a}/Gpi-1^{b} mice. Section thickness 40 µm.


frequency
20
10
GPI-1A
0 2 4 6 8 10 12 14
20
10
GPI-1AB
0 2 4 6 8 10 12 14
20
10
GPI-1B
0 2 4 6 8 10 12 14
Difference between sections (% GPI-1)

It was expected that there would be some differences between measured and predicted proportions of GPI-1AB due to experimental error. In order to determine whether, in chimaera samples, the differences were greater than could be expected as a result of experimental error, the (measured minus predicted) values for the percentage of GPI-1AB in chimaera samples were compared to the (measured minus predicted) values for the proportion of GPI-1AB in samples of mature somatic tissues from $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^b}$ mice.

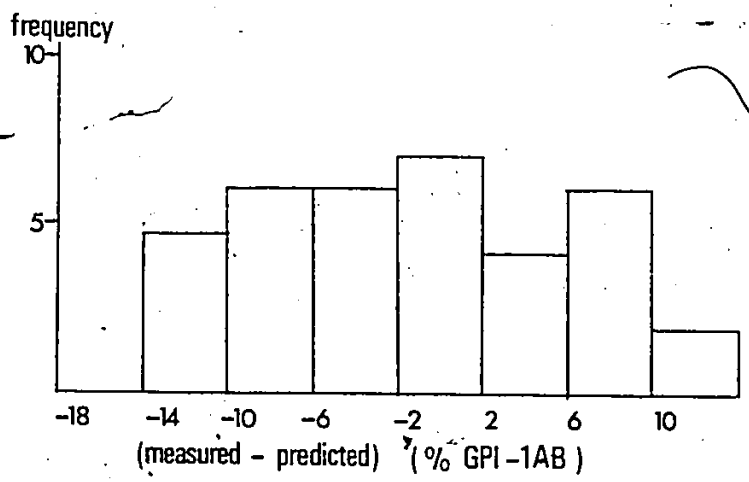
It is widely accepted that equal proportions of A and B type monomers of GPI-1 are produced and aggregate at random to produce a 25% GPI-1A: 50% GPI-1AB: 25% GPI-1B isozyme distribution in somatic tissues of mature $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^b}$ mice (Peterson and Wong, 1978). Therefore, the differences between measured and predicted proportions of GPI-1AB in samples from heterozygous mice provided estimates of the differences that could be expected to arise in chimaera samples due to experimental error.

The (measured minus predicted) values for the percentages of GPI-1AB in the heterozygous control samples are presented in Figure 8 (original data in Appendix 2). Although the mean measured proportion of GPI-1AB was 49.2%, for each single heterozygous muscle sample, the measured proportion of GPI-1AB was seldom exactly 50% of the total GPI-1 activity; over the course of this study, the measured proportions of GPI-1AB in heterozygous samples varied over a range from 36.8% to 60.8%.

Figure 8

Control (measured minus predicted) values. The differences, for samples from $\underline{Gpi\text{-}1}^{a}/\underline{Gpi\text{-}1}^{b}$ mice, between the percentage of GPI-1AB measured and the percentage of GPI-1AB predicted on the basis of random monomer aggregation are summarized in a frequency histogram. The apparent differences between the measured and predicted values in these samples are due to experimental error.

The control values for the (m-p) relative amounts of GPI-1AB appear to be normally distributed about a mean value between -1 and 0 % GPI-1AB.


frequency
10
5
-18
-14
-10
-6
-2
2
6
10
(measured - predicted) (% GPI-1AB)

GPI-1 Isozyme Distribution in Muscles from Mouse Chimaeras

1. Whole Muscles from Adult Chimaeras

A principal objective of this study was to measure the GPI-1 isozyme distribution in mosaic muscle fibres at various points along their length in order to determine whether the isozyme distributions differed along the length of the muscle cells. Initially, the distributions of GPI-1 isozymes were compared between whole muscle cross-sections taken from various points along the length of individual mosaic muscles.

In one experiment, 12 serial 30 µm thick cross-sections of the left anterior tibialis muscle of chimaera 189 (♂, age 7 months) were analyzed for GPI-1 isozymes. The differences in isozyme distribution between adjacent sections of this mosaic muscle are presented in Figure 9. Also shown in Figure 9 are the apparent differences in isozyme distribution between adjacent whole muscle cross-sections from a $Gpi\text{-}1^a/Gpi\text{-}1^b$ control animal. The differences between adjacent sections of the chimaera muscle were not significantly different from the control (i.e. error) differences ($P > 0.95$). The conclusion is, therefore, that there were not detectable differences with respect to the distribution of isozymes of GPI-1 along the length of the mosaic muscle.

In four other experiments, series of adjacent sections were not obtained but rather whole muscle cross-sections were taken from various points along the length of mosaic muscles. The differences in isozyme distribution between cross-sections separated by a variety of distances are shown in Figure 10. These differences were not larger than the apparent differences between identical control samples ($P > 0.95$). Therefore, these results also indicate that the GPI-1 isozyme profiles were uniform along the length of mosaic muscles.

Figure 9

Frequency distributions of the differences in the measured percentage of each GPI-1 isozyme band between adjacent cross-sections of the anterior tibialis muscle of chimaera 189 (solid lines) and between adjacent whole muscle cross-sections from heterozygous control mice (broken lines). Section thickness 30 μm.

The apparent differences between adjacent control sections are due to experimental error in the quantitative analysis. The differences in isozyme distribution between adjacent sections from the chimaera muscle are not larger than the differences between adjacent control sections.

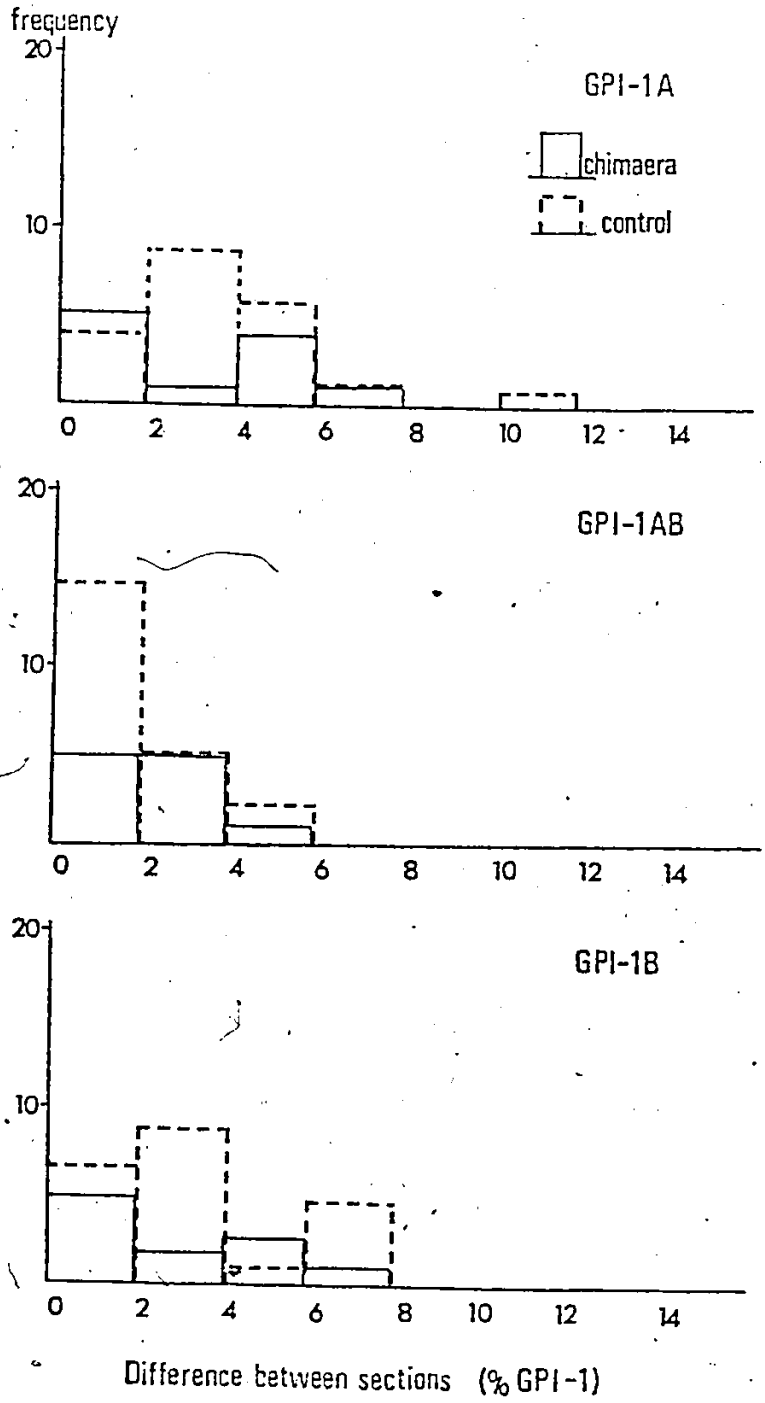
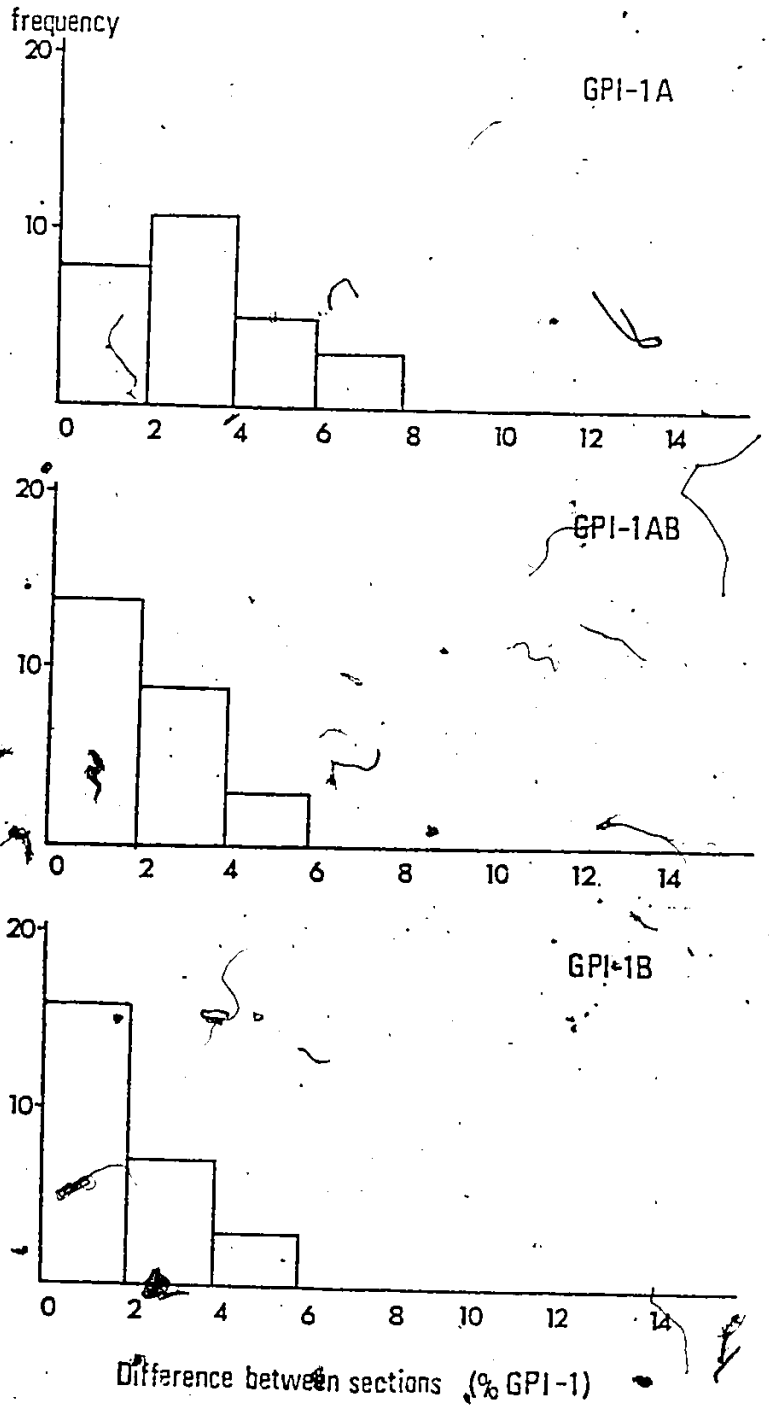

frequency
20
10
GPI-1A
chimaera
control
0 2 4 6 8 10 12 14
20
10
GPI-1AB
0 2 4 6 8 10 12 14
20
10
GPI-1B
0 2 4 6 8 10 12 14
Difference between sections (% GPI-1)

Figure 10

Frequency distributions of the differences in the measured percentage of each GPI-1 isozyme band between nonadjacent whole muscle cross-sections of mosaic muscles. Samples were taken at several points along the lengths of 2 muscles from each of 2 chimaeras (chimaera 32,♂, age 10 months, and chimaera 38,♂, age 1 yr). The sections were separated by various distances (50 to 1000 μm) and were of various thicknesses.

For each isozyme band, small differences between sections occurred more frequently than large differences, and the differences between chimaera samples are not greater than the differences between control samples (40 μm thick adjacent whole muscle cross-sections from heterozygous mice).

The results for sections of mosaic muscles separated by various distances are presented together as the numbers of pairs of sections taken at each separation distance were small. However, the differences between sections separated by as much as 1000 μm are not greater than the differences between adjacent sections.


frequency
20
10
GPI-1A
0 2 4 6 8 10 12 14
20
10
GPI-1AB
0 2 4 6 8 10 12 14
20
10
GPI-1B
0 2 4 6 8 10 12 14
Difference between sections (% GPI-1)

The finding that the GPI-1 isozyme distributions were the same at various points along the length of mosaic muscles is consistent with the hypothesis that the cytoplasm of individual mosaic muscle fibres is homogeneous with respect to GPI-1 isozymes. However, the possibility remained that, although the total isozyme distribution was identical in various cross-sections of an entire mosaic muscle, there were differentes in the isozyme distribution along the length of individual myofibres. Figure 11 is a diagram which illustrates how the distributions of isozymes obtained in adjacent whole muscle cross-sections may be identical when there are differences between adjacent sections of single muscle fibres.

To investigate directly the possibility that the GPI-1 isozymes were not uniformly distributed within individual mosaic muscle fibres, it was necessary to measure the isozyme distributions in different regions of single mosaic myofibres.

2. The Analysis of GPI-1 Isozymes in Single Muscle Fibres from Adult Mouse Chimaeras

Segments of single myofibres were isolated either by the dissection of cryostat-cut whole muscle cross-sections, or by the enzymatic disruption of whole muscles (see Materials and Methods).

Cryostat sections

To determine whether the GPI-1 isozyme distribution was different between neighbouring regions in a single mosaic muscle cell, the isozyme profiles in adjacent cross-sections of individual fibres were compared. Typical electrophoretograms are shown in Figure 12. The samples were two pairs of adjacent 30 µm thick sections.

The differences in isozyme distribution between adjacent 20, 30 and

Figure 11

Diagram illustrating how the GPI-1 isozyme distribution can be identical in 2 adjacent cross-sections of a whole mosaic muscle when the isozyme distributions are different in adjacent cross-sections of individual muscle fibres. For simplicity, only 4 muscle fibres and a few GPI-1 dimers are shown. The ovals represent myonuclei: the dark ovals are $\underline{Gpi\text{-}1}^{a}/\underline{Gpi\text{-}1}^{a}$ myonuclei and the light ovals are $\underline{Gpi\text{-}1}^{b}/\underline{Gpi\text{-}1}^{b}$ myonuclei. In each section the GPI-1 isozyme distribution is 25% GPI-1A: 50% GPI-1AB: 25% GPI-1B.

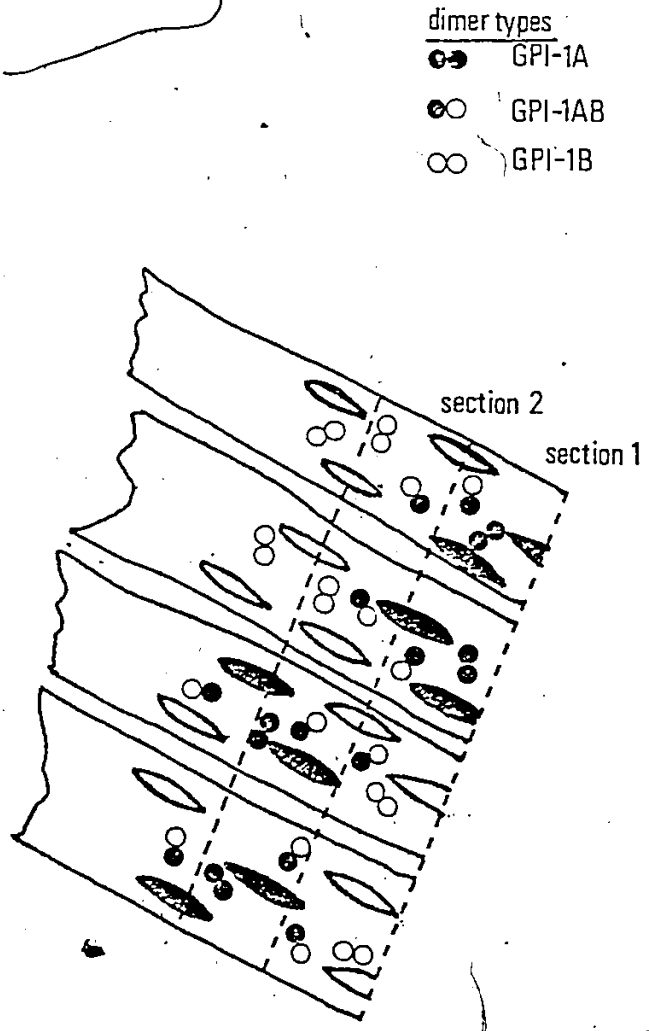
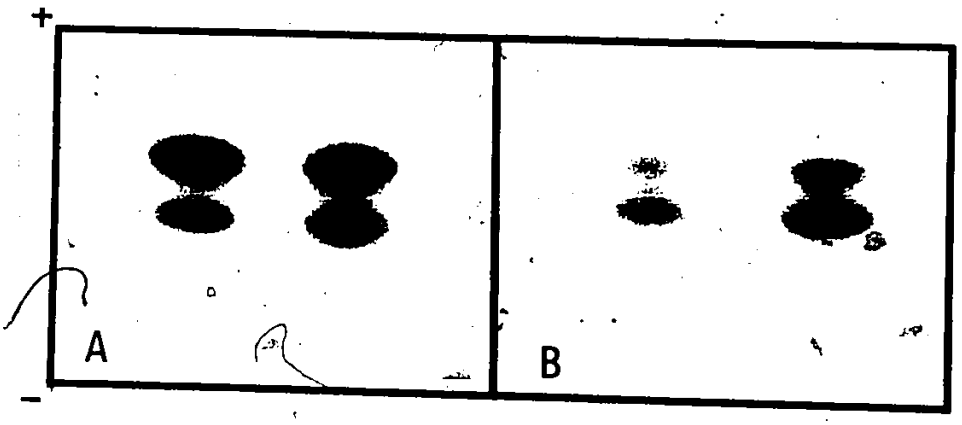

dimer types
GPI-1A
GPI-1AB
GPI-1B
section 2
section 1

Figure 12

GPI-1 phenotypes in 2 pairs of adjacent cross-sections of single muscle fibres from mouse chimaeras. Section thickness 30 µm.

a) Fibre no. 4 from the right anterior tibialis muscle of chimaera 115 (♂, age 9 mo). The isozyme distributions in the 2 samples are 61.6% GPI-1A: 34.1% GPI-1AB: 4.3% GPI-1B and 62.7% GPI-1A: 32.7% GPI-1AB: 4.6% GPI-1B.

b) Fibre no. 9 from the right anterior tibialis muscle of chimaera 142 (♀, age 4 mo). The isozyme distributions in the 2 samples are 46.0% GPI-1A: 43.4% GPI-1AB: 10.6% GPI-1B and 46.6% GPI-1A: 46.4% GPI-1AB: 7.0% GPI-1B.


+
A
B
−

40 μm thick cross-sections of single mosaic myofibres are summarized in Figures 13, 14, and 15, respectively (original data in Appendix III). At each section thickness, there were no significant differences in isozyme distribution between adjacent sections.

To investigate whether there were differences in GPI-1 isozyme distribution between nonadjacent sections of a mosaic muscle fibre, a number of single myofibres were sampled at several points along their length. For example, the GPI-1 isozyme profiles of 4 different sections of a single mosaic fibre are shown in Figure 16. The differences in isozyme distributions between single myofibre sections separated by various lengths are shown in Figure 17. There were no significant differences in isozyme profiles between sections separated by as much as 1000 μm ($P > 0.95$).

These measurements of GPI-1 isozyme distributions in single mosaic myofibres support the hypothesis that the GPI-1 isozyme profile is uniform along the length of mosaic muscle cells. However, the results of one type of control experiment indicate that it was possible that the GPI-1 recovered in sections of single muscle fibres did not come exclusively from the dissected fibre. In experiments in which muscles from $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^a}$ and $\underline{Gpi\text{-}1^b}/\underline{Gpi\text{-}1^b}$ mice were mounted together for freezing, sectioning, and dissection of single fibres, both the GPI-1A and the GPI-1B isozyme bands were recovered from some single fibres that were dissected from the boundary between the 2 muscles. The amount of 'foreign' GPI-1 in a single fibre section varied from less than 1% to approximately 40% of the total GPI-1 in the sample. These results indicate that it was possible that, in samples of single myofibres from mouse chimaeras, differences in GPI-1 isozyme distributions between various sections were masked by contaminating

Figure 13

Frequency distributions of the differences in the measured percentage of each GPI-1 isozyme band between adjacent cross-sections of single muscle fibres from chimaeras. Section thickness 20 μm. The differences between chimaera samples are not greater than the differences between adjacent cross-sections of single muscle fibres from heterozygous control mice (Figure 5).

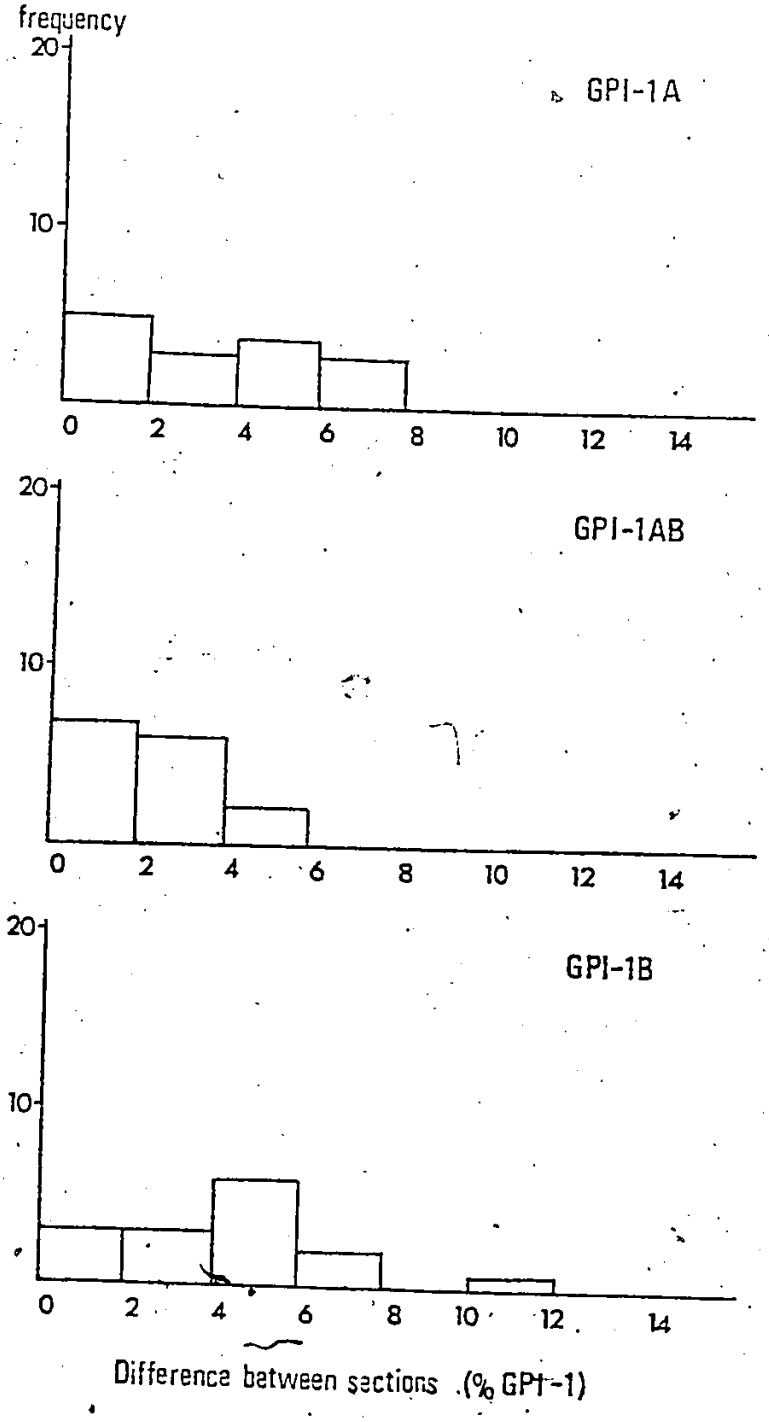

frequency
20
10
GPI-1A
0 2 4 6 8 10 12 14
20
10
GPI-1AB
0 2 4 6 8 10 12 14
20
10
GPI-1B
0 2 4 6 8 10 12 14
Difference between sections (% GPI-1)

Figure 14

Frequency distributions of the differences in the measured percentage of each GPI-1 isozyme band between adjacent cross-sections of single muscle fibres from chimaeras. Section thickness 30 µm. The differences between chimaera samples are not greater than the differences between adjacent cross-sections of single muscle fibres from heterozygous control mice (Figure 6).

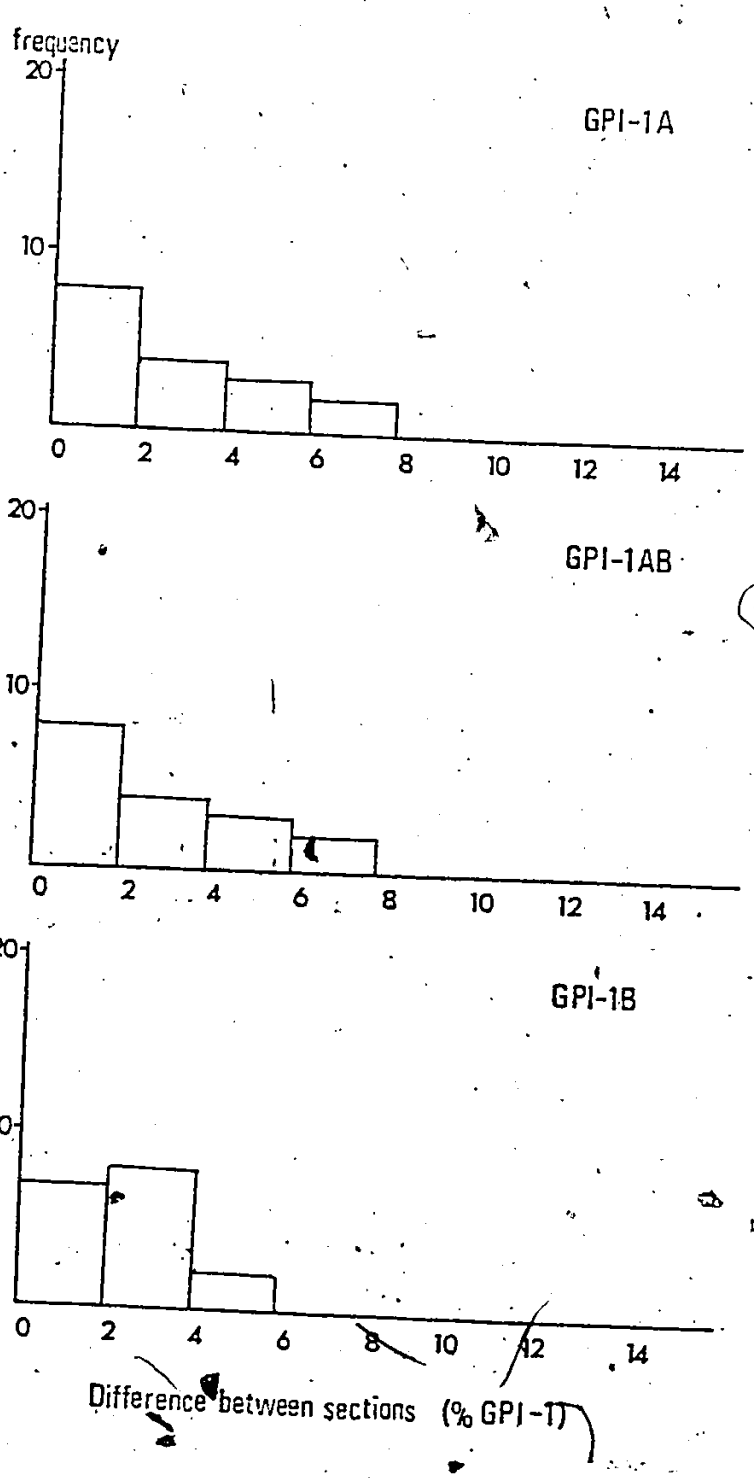

frequency
20
10
GPI-1A
0 2 4 6 8 10 12 14
20
10
GPI-1AB
0 2 4 6 8 10 12 14
20
10
GPI-1B
0 2 4 6 8 10 12 14
Difference between sections (% GPI-1)

Figure 15

Frequency distributions of the differences in the measured percentage of each GPI-1 isozyme band between adjacent cross-sections of single muscle fibres from chimaeras. Section thickness 40 µm. The differences between adjacent sections from chimaeras are not greater than the differences between adjacent cross-sections of single muscle fibres from heterozygous control mice (Figure 7).

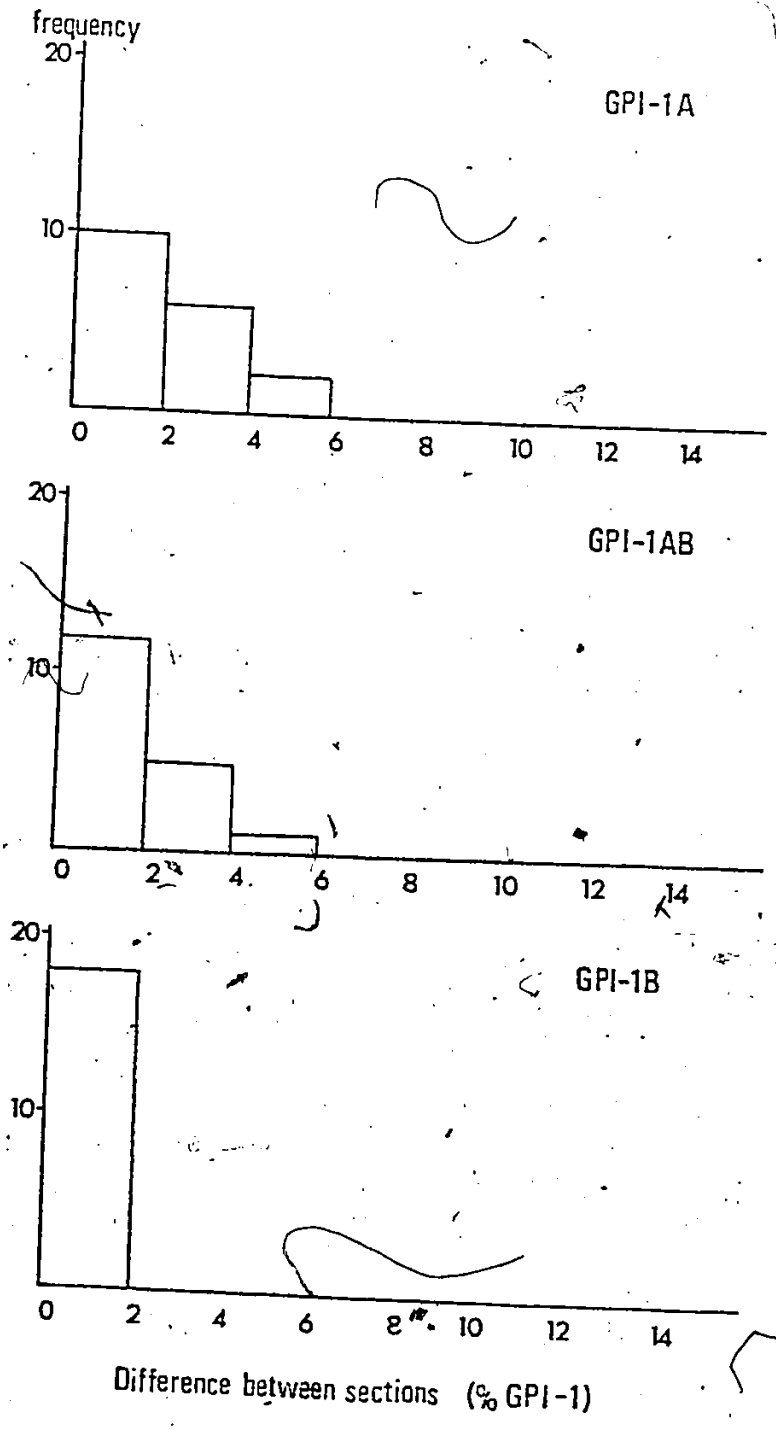

frequency
20
10
GPI-1A
0 2 4 6 8 10 12 14
20
10
GPI-1AB
0 2 4 6 8 10 12 14
20
10
GPI-1B
0 2 4 6 8 10 12 14
Difference between sections (% GPI-1)

Figure 16

GPI-1 phenotypes in 4 cross-sections of a single mosaic muscle fibre. Section thickness 30 µm.

a) Section 1, 50.1% GPI-1A: 40.0% GPI-1AB: 9.9% GPI-1B.

b) Section 3, 44.0% GPI-1A: 42.0% GPI-1AB: 14.0% GPI-1B.

c) Section 4, 46.5% GPI-1A: 42.0% GPI-1AB: 11.5% GPI-1B.

d) Section 5, 40.6% GPI-1A: 45.8% GPI-1AB: 13.6% GPI-1B.

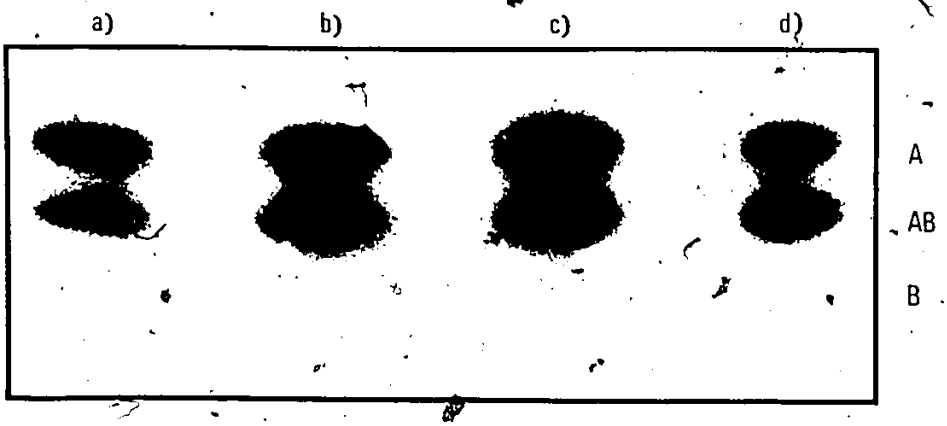
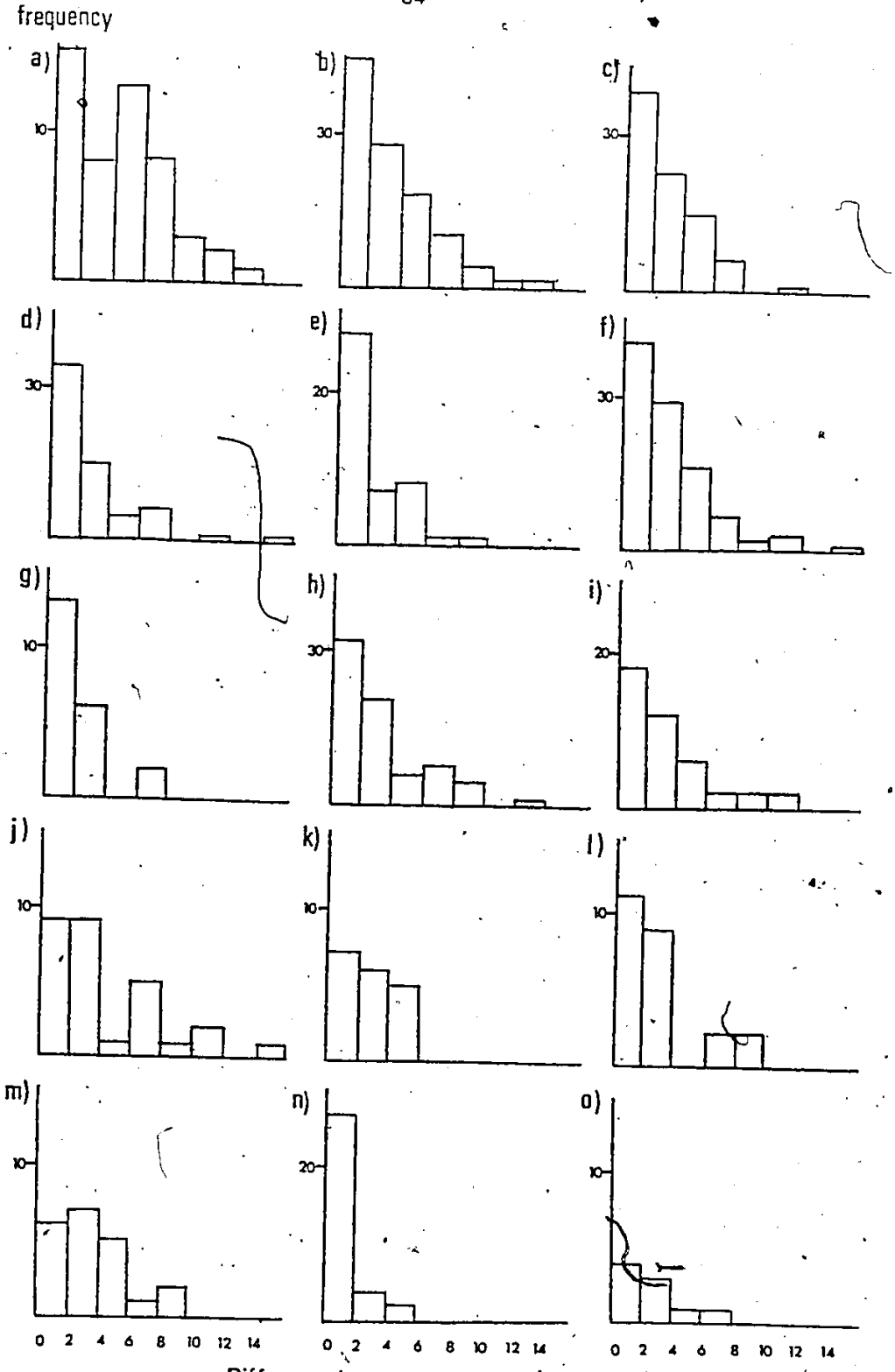

a)
b)
c)
d)
A
AB
B

Figure 17

Frequency distributions of the differences in the GPI-1 isozyme distributions between nonadjacent cross-sections of single muscle fibres from mouse chimaeras. As the shapes of the distributions are similar for all 3 GPI-1 isozymes, the results for the 3 bands are plotted together. Section thickness (in µm) and the distance between sections (in µm) respectively, are as follows: a) 20,20; b) 20,40; c) 20,60; d) 20,80; e) 20,100; f) 20,120; g) 20,140; h) 20,160; i) 20,400; j) 20,980; k) 30,30; l) 30,60; m) 30,90; n) 40,40; o) 40,80.

For each group of chimaera samples, the differences between sections of mosaic myofibres are not greater than the differences between adjacent sections of single muscle fibres from control muscles. In addition, the differences between sections of mosaic myofibres separated by longer distances are not greater than the differences between sections separated by short lengths.


frequency
a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
k)
l)
m)
n)
o)
0 2 4 6 8 10 12 14
Difference between sections (% GPI- 1)

GPI-1 from neighbouring cells. However, this possibility appears unlikely as it would require that each dissected section of a mosaic muscle fibre was contaminated with foreign GPI-1 isozymes in proportions which varied in such a way that the total isozyme distribution remained constant. It is concluded, therefore, that the total (endogenous plus contamination) GPI-1 isozyme distribution was the same in various sections of a single mosaic muscle fibre as a consequence of the homogeneous distribution of isozymes, both within the sampled fibre and within each of the neighbouring, contaminating fibres.

This conclusion is supported by the results of the second type of experiment in which single muscle fibres were obtained by the collagenase method.

Segments isolated with collagenase

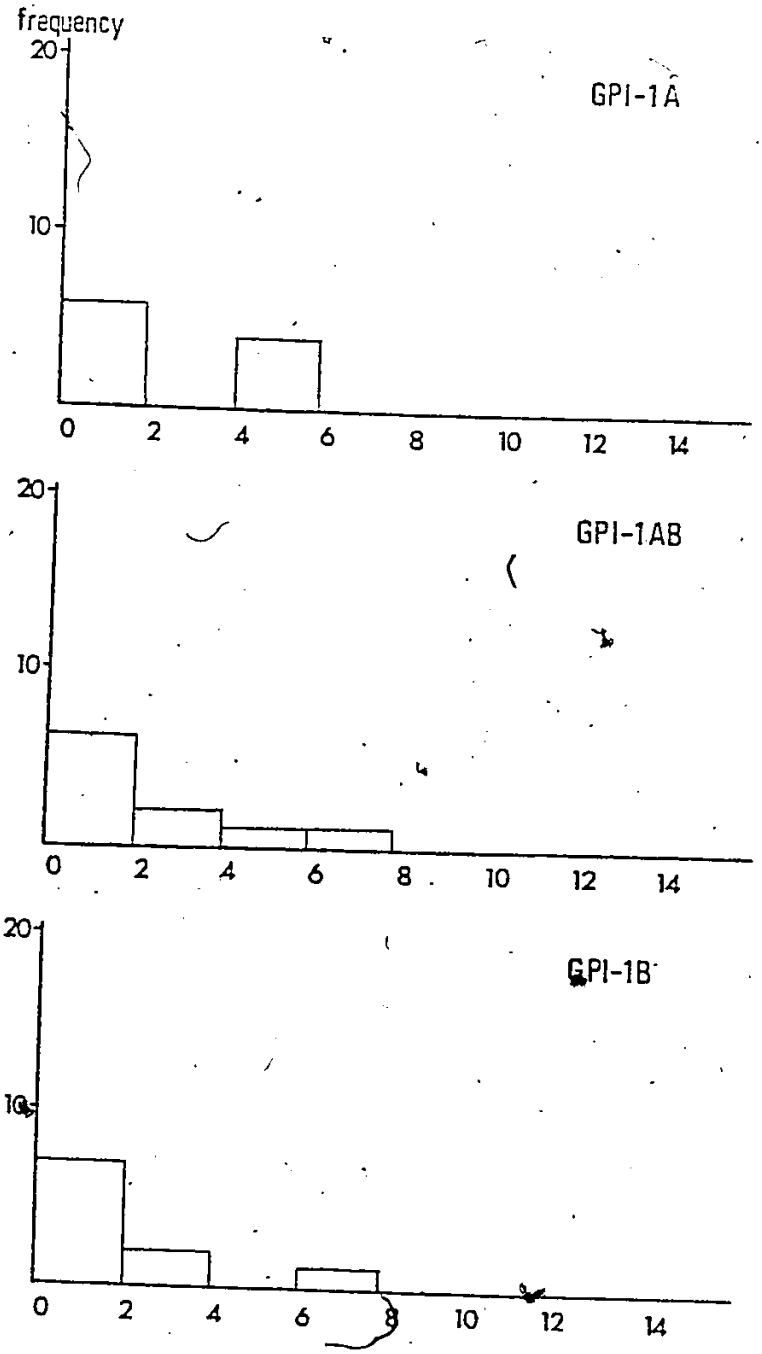
Segments of individual muscle fibres 0.5 to 2.5 mm in length were isolated by the collagenase digestion of whole muscles. This technique had the advantage of providing single cells free from contamination by other muscle cells. It was also a convenient and reliable method for sampling individual muscle fibres over lengths greater than 1 mm; the segments were cut into 2 or 3 pieces and the GPI-1 isozyme compositions of the pieces were compared.

The differences in the proportions of the three GPI-1 isozymes between adjacent 0.5 to 1.0 mm long segments from muscle fibres from 4 adult chimaeras are shown in Figure 18. There were no significant differences between adjacent segments ($P > 0.95$).

The results of these analyses of GPI-1 enzyme profiles along the lengths of whole muscles and single muscle fibres from mouse chimaeras

Figure 18

Frequency distribution of the differences in the measured percentage of each GPI-1 isozyme band between adjacent 0.5 to 1.0 mm long segments of muscle fibres from adult chimaeras. The differences between these chimaera samples are not greater than the differences between adjacent cross-sections of single muscle fibres from control mice (control section thickness 40 µm.).


frequency
20
10
GPI-1A
0 2 4 6 8 10 12 14
20
10
GPI-1AB
0 2 4 6 8 10 12 14
20
10
GPI-1B
0 2 4 6 8 10 12 14
Difference between sections (% GPI-1)

indicate that the isozymes were homogeneously distributed along the length of muscle cells from adult chimaeras.

3. The Analysis of GPI-1 Isozymes in Single Muscle Fibres from Immature Mouse Chimaeras

The turnover rates for both RNA and many proteins are more rapid in myofibres of young animals, and the cytoplasm of immature muscle cells is densely packed with ribosomes (see Introduction). In view of these and other differences between the myofibres of mature and immature animals, it was of interest to investigate the possibility that the isozymes of GPI-1 are not homogeneously distributed within mosaic myofibres from young mouse chimaeras. Therefore, some preliminary experiments were done to determine the GPI-1 isozyme profiles in adjacent segments of single muscle fibres from embryonic, newborn, and young chimaeras.

The collagenase method was used to obtain segments of single muscle fibres from immature mice as the amount of GPI-1 in cryostat cross-sections of single young muscle cells was too low to be detected on starch gels. The GPI-1 isozyme distributions in adjacent segments of individual myofibres from newborn, one week old, and two week old chimaeras are given in Table 1. When the differences between adjacent segments at the 3 ages were grouped and compared to the differences between control sections, the differences between adjacent segments from chimaeras were not significant ($P > 0.95$). (Single muscle cells from embryonic day 17 chimaeras were obtained but did not contain detectable amounts of GPI-1).

These results indicate that the GPI-1 isozyme distribution is homogeneous in mosaic muscle fibres of newborn mice. Thus, the widespread distribution of the gene products of different myonuclei occurs in immature myofibres as well as in the fibres of adult mice.

TABLE 1. DISTRIBUTION OF GPI-1 ISOZYMES IN ADJACENT SEGMENTS OF SINGLE MUSCLE FIBRES FROM YOUNG CHIMAERAS.

SOURCE	FIBRE NO	SEGMENT NO.	% GPI-1 A	AB	B
Chimaera 214, newborn	1	1	12.4	37.1	50.5
		2	5.0	38.1	56.9
		3	7.1	41.0	51.9
	2	1	0.8	21.6	77.6
		2	1.4	24.8	73.8
	3	1	1.0	13.6	85.4
		2	n.d*	14.5	85.5
Chimaera 204, 1 wk old	1	1	72.1	23.3	4.6
		2	68.4	29.0	2.6
Chimaera 205, 1 wk old	1	1	29.3	43.7	27.0
		2	35.8	38.8	25.4
Chimaera 206, 2 wk old	1	1	25.6	49.9	24.5
		2	31.7	43.7	24.6
	2	1	54.9	38.6	6.5
		2	51.2	40.0	8.8
	3	1	44.1	41.3	14.6
		2	45.7	41.2	13.1

*n.d: none detectable

Aggregation of Monomers of GPI-1 in Mosaic Muscle Fibres

In order to gain some insight into how the different types of GPI-1 dimers become homogeneously distributed within mosaic myofibres, the question was whether the isozyme distributions within each single muscle fibre reflected random or nonrandom aggregation. If the monomers of GPI-1 had remained localized in territories around the nuclei that directed their synthesis until after dimer assembly, then it was expected that the isozyme distributions would not have reflected random aggregation; rather, there would have been relatively little GPI-1AB in samples, indicating that there had been very little interaction between different monomer types. However, if the monomers of GPI-1 had been homogeneously distributed prior to the assembly of dimers, then the ratio of the 3 types of dimers would have reflected random aggregation.

The first step in this analysis was, for each sample, to calculate, on the basis of the measured isozyme distribution, the percentage of total GPI-1 activity that would have been present in the heteropolymer band if all monomers in the sample had aggregated at random (see Materials and Methods). The next step was to calculate, both for chimaera and heterozygote control samples, the difference between the proportion of total GPI-1 activity that was actually measured in the heteropolymer band and the proportion of heteropolymer predicted on the basis of random aggregation. It is known that monomer aggregation is random in the heterozygote control samples. Therefore, the (measured minus predicted) values, (m-p), for control and chimaera samples were compared in order to determine whether the amount of GPI-1AB that was present in chimaera samples was significantly different from the amount that would have resulted from random monomer aggregation.

1. Monomer Aggregation in Adult Chimaeras Single Cells

The (m-p) values for 7 groups of single muscle fibre samples from adult chimaeras are shown in Figure 19. The samples consisted of 20, 30 and 40 μm thick cross-sections of single cells and 0.5 to 1.0 mm long segments of individual fibres. The (m-p) values were not different between chimaeras and heterozygote control samples for any of the groups of mosaic myofibre samples ($P > 0.95$).

These results support the hypothesis that the GPI-1 dimers present in cross-sections and segments of single mosaic muscle fibres are assembled by random aggregation in a homogeneous monomer pool.

Whole muscle sections

To test whether it was possible to detect differences between the measured and predicted relative amounts of GPI-1AB in samples in which it was expected that all monomers had not aggregated at random, the (m-p) values were calculated for whole muscle cross-sections from mouse chimaeras. Since each section contained segments of hundreds of muscle cells as well as many mononucleated non-muscle cells, and since every cell had been an individual compartment with respect to the assembly of GPI-1 dimers, it was very unlikely that the measured proportion of GPI-1AB would be equal to the amount predicted on the basis of random aggregation of all the monomers present in the sample. The (m-p) values for samples of whole muscle cross-sections from chimaeras are presented in Figure 20. When this particular group of experimental (m-p) values was compared to the control values, the relative amounts of GPI-1AB measured in the whole muscle sections were significantly less than the amounts predicted ($P < 0.01$). Therefore, the conclusion is that monomer aggregation was nonrandom in the whole muscle sections.

Figure 19

Experimental (m-p) values. The (m-p) values for single muscle fibre samples from adult chimaeras are summarized in frequency histograms. The sources of the samples are as follows: a) chimaera 115 (♂, age 11 mo), 20 µm thick sections; b) chimaera 142 (♀, age 4 mo), 20 µm thick sections; c) chimaera 145 (♂, age 1 yr), 20 µm thick sections; d) chimaera 149 (♀, age 10 wk), 20 µm thick sections; e) various chimaeras, 30 µm thick sections; f) various chimaeras, 40 µm thick sections; g) various chimaeras, collagenase-isolated segments 0.5 to 1.0 mm in length.

In all these groups of samples, the most frequently occurring (m-p) values are close to zero, as in the control samples (see Figure 8). If there had been preferential aggregation of like monomers in the mosaic muscle cells, the distributions of (m-p) values would be shifted to the left (see, for example, Figure 20).

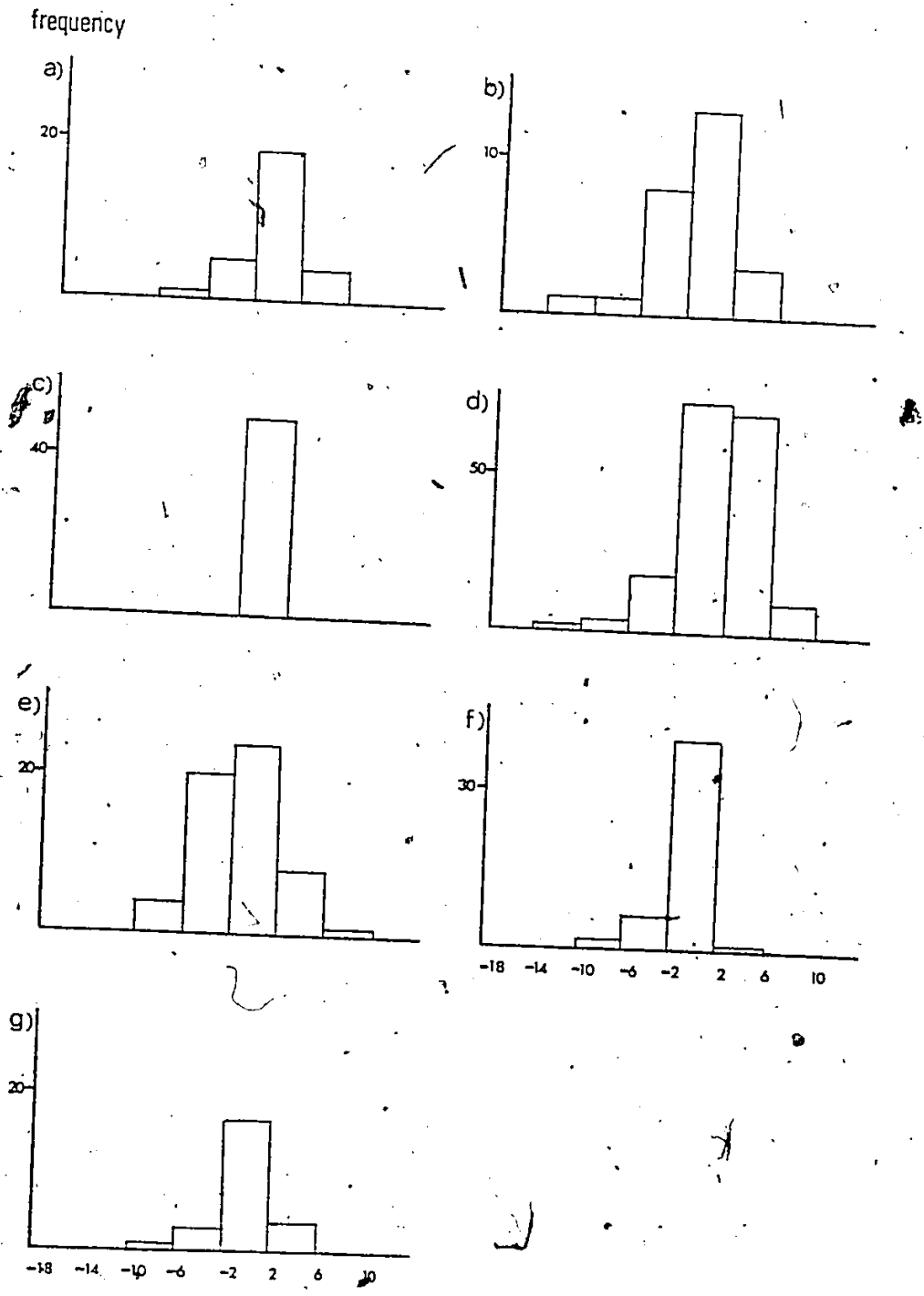
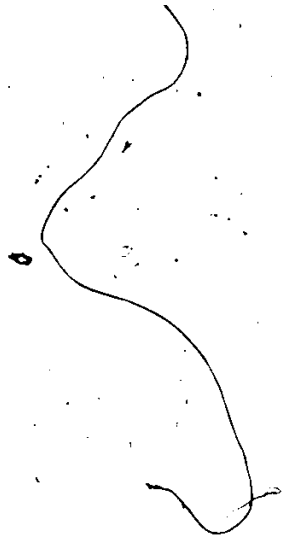
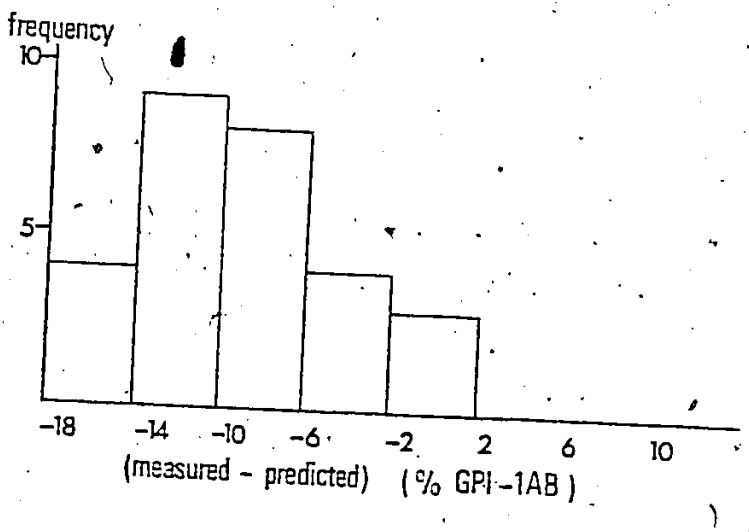

frequency
a)
20
b)
10
c)
40
d)
50
e)
20
f)
30
-18 -14 -10 -6 -2 2 6 10
g)
20
-18 -14 -10 -6 -2 2 6 10
(measured - predicted) (% GPI-1AB)

Figure 20

Frequency distribution of the (m-p) values in whole muscle samples. The distribution is shifted to the left when compared to the distribution of control (m-p) values (Figure 8); in cross-sections of whole mosaic muscles, the (m-p) values are large negative numbers, indicating that there has been some preferential aggregation of like monomers in these samples.




frequency
10
5
−18
−14
−10
−6
−2
2
6
10
(measured − predicted) (% GPI−1AB)

Fibre types

There are many differences in morphological and biochemical characteristics between muscle fibres with different histochemical staining properties. For example, proteins turn over more rapidly in muscles which contain a majority of fibres that stain intensely for enzymes of oxidative metabolism (e.g., Goldberg, 1967). Oxidative fibres are also richer in large mitochondria and have wider Z-lines than glycolytic fibres (e.g., Gauthier and Dunn, 1973). In view of the many differences between histochemical fibre types, it was of interest to examine the (m-p) values for the relative amount of GPI-1AB in mosaic muscle fibres of different histochemical types; it was possible that significant differences between measured and predicted values would be revealed within one or another group of experimental samples when all the fibres within each group were of the same type.

The (m-p) values for the percentage of GPI-1AB in mosaic muscle fibres of high, intermediate and low succinic dehydrogenase (SDH) activities are presented in Figure 21. Fibres which stain darkly for SDH activity are small diameter, slow twitch, oxidative fibres while those which stain lightly are large diameter, fast twitch, glycolytic fibres. The different fibre types were not significantly different from one another nor from the control group with respect to the (m-p) values for the proportion of GPI-1AB; in all mosaic muscle fibres monomer aggregation had been random.

2. Monomer Aggregation in Immature Chimaeras

The differences between measured and predicted relative amounts of GPI-1AB in collagenase isolated segments of single muscle fibres from newborn, one week old, and two week old chimaeras are presented in Figure 22. When these data were compared to the control (m-p) values, the results

Figure 21

Frequency distributions of (m-p) values in cross-sections of single mosaic muscle fibres of high (a), intermediate (b), and low (c) succinic dehydrogenase activity. In all these histochemical fibre types the most frequently occurring (m-p) values are close to zero, as in control samples.

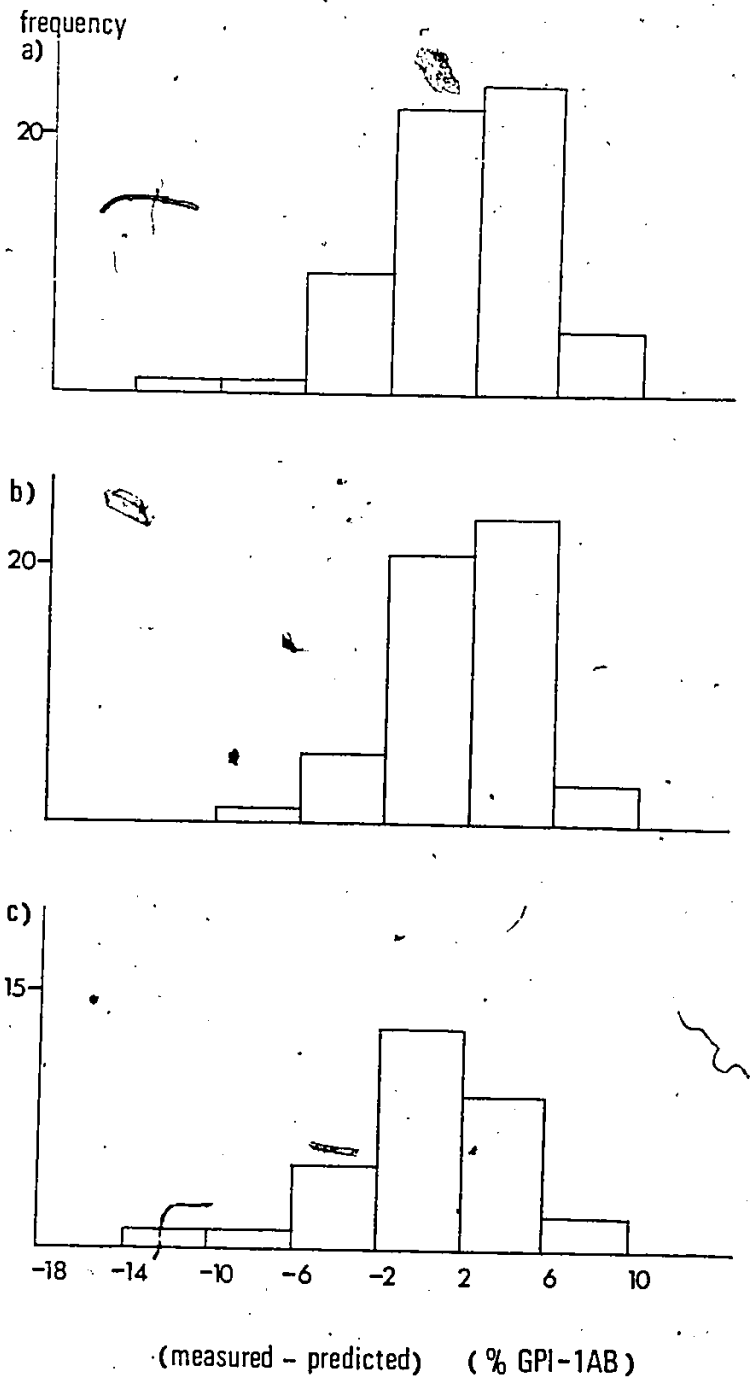
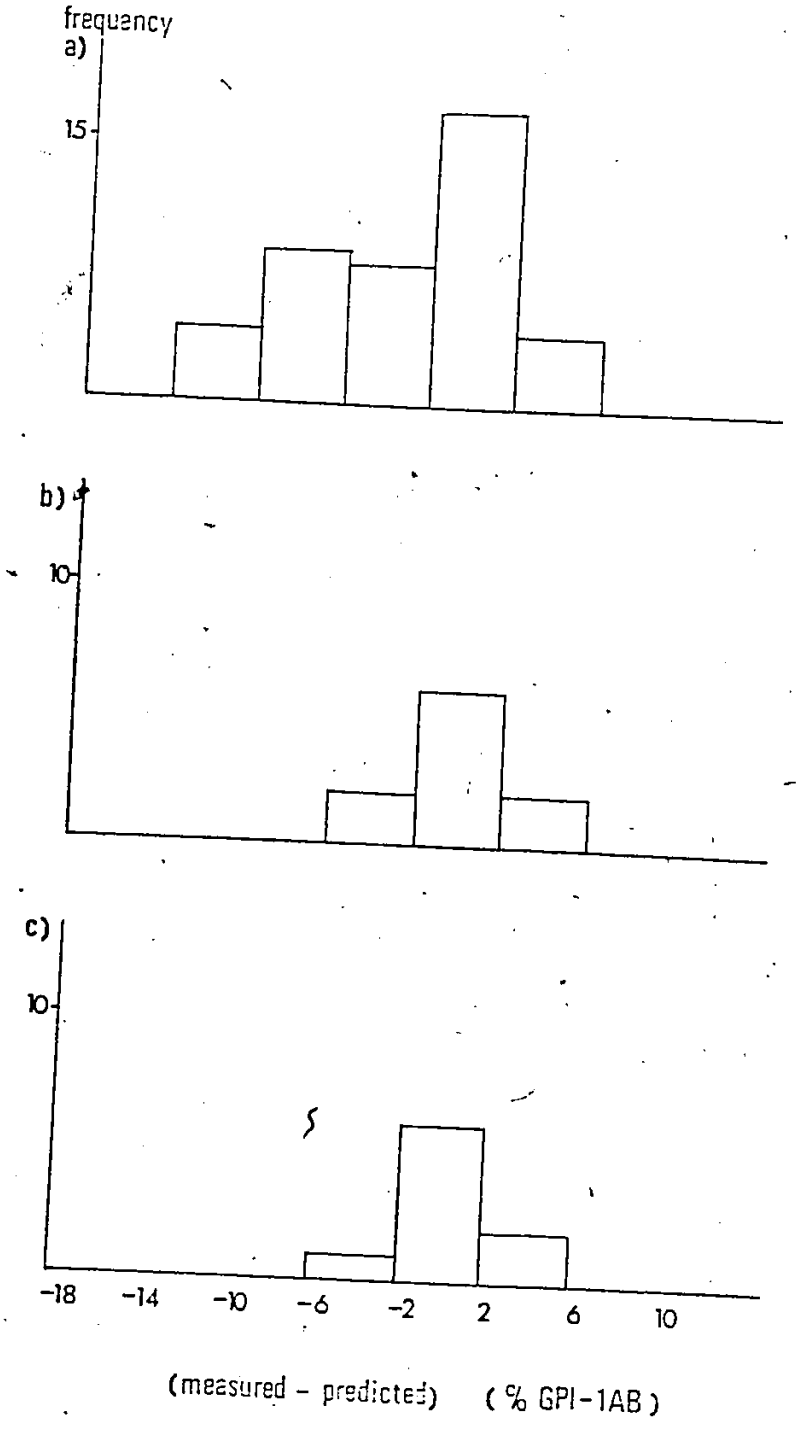

frequency
a)
20
b)
20
c)
15
-18
-14
-10
-6
-2
2
6
10
(measured - predicted) (% GPI-1AB)

Figure 22

Frequency distributions of (m-p) values in segments of single mosaic myofibres from young chimaeras. The ages were as follows: a) newborn; b) 1 wk old; c) 2 wk old.

In these samples from young chimaeras the most frequently occurring (m-p) values are close to zero, as in control samples.


frequency
a)
15
b)
10
c)
10
-18
-14
-10
-6
-2
2
6
10
(measured - predicted) (% GPI-1AB)

indicate that monomer aggregation had been random in the myofibres of these young chimaeras. In addition, it has been shown that GPI-1AB is present in the muscles of chimaeras at embryonic day 16 (D.P. Cross, unpublished results). These findings indicate that interaction occurs between the gene products of different myonuclei very early in myogenesis, and that a homogeneous pool containing GPI-1 monomers encoded by many myonuclei is already established in myofibres at the time of birth.

In summary, the results presented in this section indicate that, in skeletal muscle fibres of the mouse, the dimers of GPI-1 are assembled by random aggregation of the monomers encoded by multiple myonuclei. This finding, together with the results which show that the GPI-1 isozyme profile is uniform along the length of each mosaic muscle fibre, could indicate that the distribution of GPI-1 monomers encoded by different myonuclei is homogeneous throughout each myofibre prior to dimer formation. Alternatively, it is possible that all monomers are synthesized, randomly mixed, and aggregate within a single site, for example, in the region of the motor endplate (Gauthier & Dunn, 1973), and that these events are followed by homogeneous distribution of assembled dimers throughout the cell. Whether a single site or multiple sites of dimer assembly exist(s), the results obtained in these experiments provide direct evidence that each region of cytoplasm in a muscle fibre has access to the gene products of multiple myonuclei.

QUALITATIVE ANALYSIS OF MOD-2 ISOZYMES

The third major aim of this study was to determine whether heteropolymers of a nuclear-coded oligomeric protein, mitochondrial malic enzyme (MOD-2), are present in the mitochondria of mosaic muscle fibres from $\underline{Mod\text{-}2^a}/\underline{Mod\text{-}2^a} \leftrightarrow \underline{Mod\text{-}2^b}/\underline{Mod\text{-}2^b}$ mouse chimaeras. The finding of MOD-2 heteropoly-

mers in mitochondria of mosaic myofibres would indicate that monomers of MOD-2 widely distributed in muscle cells both before their incorporation into mitochondria and before their assembly into oligomers.

Electrophoretic and Staining System for MOD-2

1. Resolution of Isozymes

The separation and resolution of MOD-2 isozymes obtained in the starch gel electrophoretic system are shown in Figure 23. Single fast MOD-2A, Figure 23c) and single slow (MOD-2B, Figure 23a) bands were obtained from SM/J ($Mod\text{-}2^a/Mod\text{-}2^a$) and C57BL/6J ($Mod\text{-}2^b/Mod\text{-}2^b$) tissues, respectively.

The pattern of MOD-2 isozyme activity in skeletal muscles of SM/J x C57BL/6J F_1 mice is shown in Figure 23d. It is thought that equal numbers of A and B MOD-2 monomers are produced and aggregate into tetramers at random in $Mod\text{-}2^a/Mod\text{-}2^b$ heterozygous cells. Accordingly, the 5 tetramer types should distribute in the ratio of 1AAAA: 4AAAB: 6AABB: 4ABBB: 1BBBB, with the 3 heteropolymers having intermediate electrophoretic mobilities (Shows et al., 1970). Although the observed pattern of activity appears to be consistent with the theoretical distribution, the 5 isozyme bands were not clearly resolved on the starch gels. However, the resolution obtained in the electrophoretic system did allow the distinction between samples which contained only fast and slow bands of MOD-2 (Figure 23b) and samples which contained MOD-2 heteropolymers (Figure 23d). Therefore, it was possible to use the system to determine whether or not MOD-2 heteropolymers were present in samples from mouse chimaeras.

2. Conditions of Heteropolymer Formation

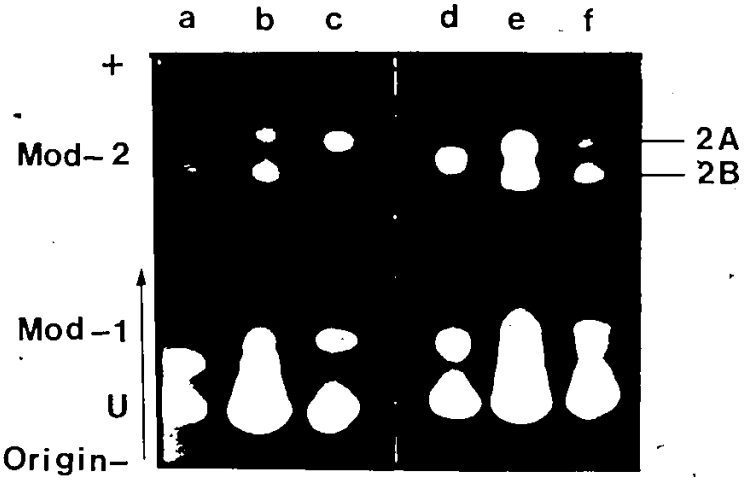
Samples prepared from a mixture of skeletal muscle sections from mice of $Mod\text{-}2^a/Mod\text{-}2^a$ and $Mod\text{-}2^b/Mod\text{-}2^b$ genotypes yielded only the fast and slow homopolymer bands of MOD-2 (Figure 23b, 23f). Similarly, only the

Figure 23

MOD-2 phenotypes in whole cell extracts from mouse skeletal and cardiac muscles. Starch gel electrophoresis in Tris-citrate, pH 8.6. Under these conditions MOD-2 migrates toward the anode. Cytoplasmic malic enzyme (MOD-1) also migrates anodally but with lower electrophoretic mobility in this system. Additional bands seen near the origin and labeled U in these electrophoretograms, are active in the absence of L-malate and were shown not to be isocitrate dehydrogenase either.

Sources of the samples are as follows:

a) C57BL/6J; b), f) 50:50 mix SM/J:C57BL/6J; c) SM/J; d) C57BL/6J X SM/J F_1; e) chimaera 115, cardiac muscle.


a
b
c
d
e
f
+
Mod–2
Mod–1
U
Origin–
2A
2B

2 homopolymer bands were present in extracts from the mononucleated cells of the cardiac muscle of an SM/J ↔ C57BL/6J mouse chimaera (Figure 23e). Thus, MOD-2 heteropolymers were not generated during extraction and electrophoresis of the enzyme.

MOD-2 Phenotype of Muscles from Mouse Chimaeras

In order to investigate whether individual mitochondria have access to MOD-2 monomers encoded by one or by multiple myonuclei, the MOD-2 phenotypes were determined for muscles from $\underline{Mod\text{-}2^a/Mod\text{-}2^a} \leftrightarrow \underline{Mod\text{-}2^b/Mod\text{-}2^b}$ mouse chimaeras. Examples of electrophoretograms of MOD-2 activity in extracts from skeletal muscles of four chimaeras and nonmosaic control mice appear in Figures 24 and 25.

1. Whole Cell Extracts

There was clear evidence of MOD-2 heteropolymers in the whole cell extracts of most of the mosaic muscles. Heteropolymers were recovered both from the relatively mitochondria-rich soleus muscles (Figure 25n) and from the predominantly glycolytic gastrocnemius muscle fibres (Figure 25o) of chimaeras. These results show that there are opportunities for MOD-2 monomers encoded by different myonuclei to interact in the multinucleated skeletal muscle fibres.

2. Mitochondrial Extracts

In mouse kidney cells there is no MOD-2 activity in the cytoplasm; all the functional MOD-2 tetramers are located in mitochondria (Bernstine, 1979). However, it is not yet known whether there is MOD-2 activity in the cytoplasm of muscle cells.

To investigate the possibility that MOD-2 heteropolymers were present in the cytoplasm of mosaic muscle cells but were not present in mitochondria, mitochondria were isolated from mosaic muscles and mitochondrial extracts were analyzed for MOD-2 isozymes. Although much of the MOD-2 activity was

Figure 24

MOD-2 phenotypes in whole cell extracts from skeletal muscles of various mice. Sources of the samples are as follows:

a),j),o),w) 50:50 mix SM/J:C57BL/6J; b),d) chimaera 105 (♂, age 2 mo), semitendinosus muscle; c),h),l),m),s),x),b') SM/J X C57BL/6J F_1; e),t), u),z),e') C57BL/6J; f),v) 75:25 mix SM/J:C57BL/6J; g) chimaera 115 (♂, age 11 mo), right gastrocnemius muscle; i),p),q),c') SM/J; k) chimaera 115, left gastrocnemius; n) chimaera 115, left triceps; r) chimaera 139 (♀, age 6 wk), right anterior tibialis muscle; v) chimaera 140 (♂, age 1 yr), left gastrocnemius; a') chimaera 142 (♀, age 4 mo), left gastrocnemius; d'),f') chimaera 149 (♀, age 4 mo), right gastrocnemius.

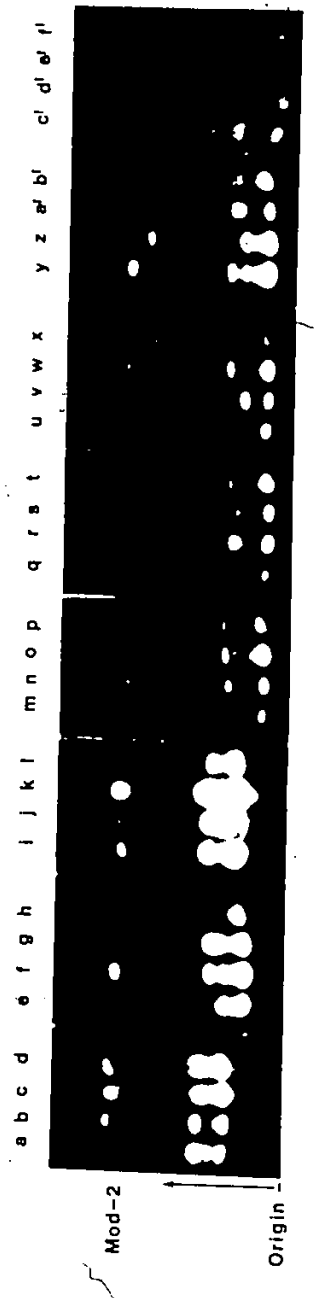
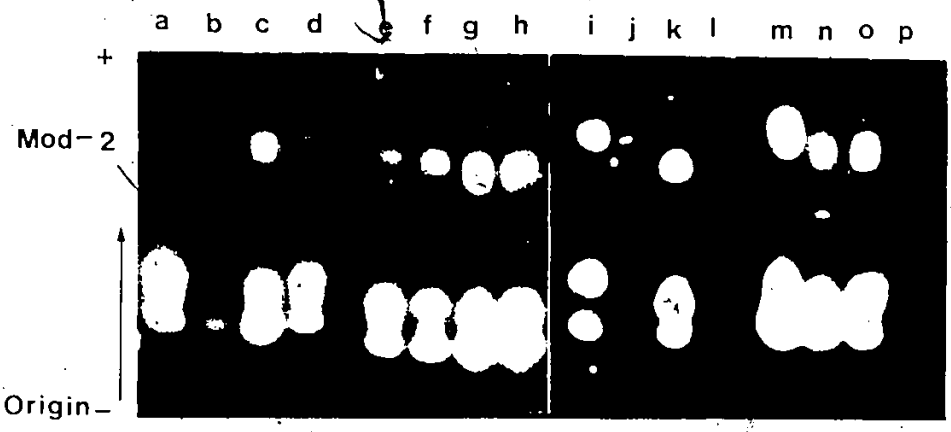

a b c d e f g h i j k l m n o p q r s t u v w x y z a' b' c' d' e' f'
Mod-2
Origin

Figure 25

MOD-2 phenotypes in whole cell (a,c,d,e,f,g,h,i,k,l,m,n,o) and mitochondrial (b,j,p) extracts of mouse skeletal muscles. Sources of the samples are as follows: a),d),e) approximately 50:50 mixture of SM/J and C57BL/6J; b),f),h) chimaera 105, right biceps femoris muscle; c),g) SM/J X C57BL/6J F_1; i) SM/J; j),p) chimaera 149, left gastrocnemius; k) C57BL/6J; l),o) chimaera 149, right gastrocnemius; m) chimaera 115, left soleus; n) chimaera 149, right soleus.

The staining intensity of mitochondrial samples is reduced, and MOD-2 activity is not visible in most photographs of the gels. However, there were definitely heteropolymers in the mitochondrial extracts from chimaera 149. The MOD-2 isozyme distributions in both the mitochondrial and whole cell extracts from the right biceps femoris muscle of chimaera 105 were skewed and composed mainly of the fast band(s).


a b c d e f g h i j k l m n o p
+
Mod-2
Origin-

lost in the isolation procedure, heteropolymers of MOD-2 were indeed present in the extracts (Figure 25j,p, and results not shown). Furthermore, when both mitochondrial and whole-cell extracts were prepared from a single skeletal muscle of a chimaera, each extract contained a similar pattern of MOD-2 activity (Figure 25b,f). Therefore, it is likely that the distributions of MOD-2 isozymes in the whole-cell extracts of mosaic muscles were identical to the MOD-2 distributions in mitochondria.

Taken together, these results indicate that MOD-2 monomers encoded by different myonuclei can be incorporated into a single mitochondrion of a muscle fibre. Thus MOD-2 monomers must be widely distributed in a muscle cell both before their incorporation into oligomers and before their uptake into mitochondria.

COMPARISON OF GPI-1 AND MOD-2 PHENOTYPES IN MUSCLES FROM MOUSE CHIMAERAS

The comparison of GPI-1 and MOD-2 isozyme distributions in muscles from SM/J ($\underline{Gpi\text{-}1}^a/\underline{Gpi\text{-}1}^a$, $\underline{Mod\text{-}2}^a/\underline{Mod\text{-}2}^a$) ↔ C57BL/6J ($\underline{Gpi\text{-}1}^b/\underline{Gpi\text{-}1}^b$, $\underline{Mod\text{-}2}^b/\underline{Mod\text{-}2}^b$) mouse chimaeras can provide some insight into the question of the functional equivalence of myonuclei. In general, the distributions of mitochondrial MOD-2 isozymes paralleled those of the cytosolic GPI-1 isozymes (Table 2). In samples which contained a heterozygote-like distribution of GPI-1 isozymes, the MOD-2 isozyme distribution was also similar to the heterozygote pattern (Figure 24k). In muscles where the GPI-1 patterns were skewed, indicating a preponderance of SM/J or C57BL/6J GPI-1 monomers, the MOD-2 distributions were also skewed (Figure 24r). Thus, it appeared that the nuclei that were active in directing the synthesis of GPI-1 were also responsible for MOD-2 synthesis.

These results provide indirect evidence for the functional equivalence of the myonuclei within skeletal muscle.

TABLE 2. COMPARISON OF GPI-1 AND MOD-2 PHENOTYPES IN MUSCLES FROM ADULT CHIMAERAS

CHIMAERA	MUSCLE	% GPI-1 A	% GPI-1 AB	% GPI-1 B	MOD-2 FIGURE	DISTRIBUTION DESCRIPTION
105	right biceps femoris	87	13	n.d*	25b	Mainly AAAA
115	heart	71	n.d	29	23e	AAAA ≃ BBBB
115	left triceps	52	35	13	24n	skewed toward AAAA
115	left soleus.	60	32	8	25m	skewed toward AAAA
115	left gastrocnemius	50	35	15	24k	skewed toward AAAA
115	right gastrocnemius	46	37	17	24g	skewed toward AAAA
139	right anterior tibialis	86	13	1	24r	mainly AAAA
140	left gastrocnemius	11	25	64	24v	mainly BBBB
142	left gastrocnemius	36	36	28	24a	heterozygote-like
149	right gastrocnemius	28	34	38	24d	heterozygote-like
149	right soleus	14	32	54	25n	heterozygote-like

*n.d: not detectable

DISCUSSION

DISCUSSION

The purpose of this study was to determine whether the mammalian skeletal muscle fibre is a functional syncytium in which the myonuclei contribute to a common pool of gene products, or if the myofibre is subdivided into territories each under the control of the local myonucleus. The most direct approach that was taken to the question was the investigation of the distribution of genetically determined electrophoretic variants of the cytosolic enzyme glucosephosphate isomerase, GPI-1, along the length of muscle fibres that contained two genetically distinct types of myonuclei: myonuclei of one type encoded monomers of the slow electrophoretic variant (GPI-1A), and myonuclei of the other type encoded monomers of the fast variant (GPI-1B). As genetically different myonuclei were distributed along the length of each mosaic myofibre, the type of GPI-1 present in different regions along the fibre length would have varied if each myonucleus controls a territory with respect to the maintenance of GPI-1. However, the profile of GPI-1 isozymes would have been uniform along the fibre length if the products of different myonuclei are distributed throughout the fibre.

The results obtained in this study indicate that the isozymes of GPI-1 were uniformly distributed along the length of mosaic muscle fibres and, therefore, each myonucleus does not contribute GPI-1 to only a limited part of a myofibre.

To examine further the functional organization in skeletal myofibres, it was investigated whether the precursors of oligomeric enzymes remain localized around the nuclei that directed their synthesis prior to the assembly of complete protein molecules, or if oligomer formation occurs

at sites that have access to enzyme monomers encoded by different myonuclei. Two approaches were taken to this question:

1) The fact that functional GPI-1 is a dimer provided the opportunity to determine whether, within segments of mosaic myofibres, the proportions of the different types of GPI-1 reflected random or nonrandom aggregation of all the GPI-1 monomers present in the sample. If the monomers or their precursors are homogeneously distributed prior to dimer formation, the isozyme profiles in mosaic cells would have reflected the random and equiprobable interaction of the different monomer types during aggregation. However, if A monomers had been prevalent at some sites of dimer assembly and B monomers had been prevalent at others, the isozyme distributions would have reflected nonrandom aggregation.

The results presented here support the hypothesis that GPI-1 monomers encoded by multiple myonuclei are uniformly distributed at all sites of dimer assembly; the observed distributions of GPI-1 isozyme activities reflected the random aggregation of different monomer types.

2) It was also investigated whether the precursors of an oligomeric protein that is localized in a specific intracellular compartment are widely distributed before oligomer formation. The specific question was whether heteropolymers of the nuclear-coded mitochondrial matrix protein malic enzyme (MOD-2) were present in the mitochondria of myofibres in which each nucleus encoded only one type of MOD-2 monomer and different nuclei encoded different monomer types (that is, in mosaic myofibres from $\underline{\text{Mod-2}}^{a}/\underline{\text{Mod-2}}^{a} \leftrightarrow \underline{\text{Mod-2}}^{b}/\underline{\text{Mod-2}}^{b}$ mouse chimaeras). Although the mechanism of incorporation of nuclear-coded mitochondrial proteins is poorly understood, it was expected that heteropolymers of MOD-2 would

have been present in the mitochondria of mosaic myofibres only if monomers are widely distributed before both oligomer formation and localization of monomers/oligomers in mitochondria. If heteropolymers had not been present in the mitochondria of mosaic muscle cells, then one possibility would be that each myonucleus is linked directly and exclusively to the mitochondria in its vicinity with respect to the maintenance of MOD-2; by this scheme, only a single type of MOD-2 monomer would be available to individual mitochondria.

The present results show that heteropolymers of MOD-2 were present in the mitochondria of mosaic myofibres. Therefore, monomers of MOD-2 must be widely dispersed both before they are incorporated into mitochondria and before they are assembled into oligomers.

Taken together, the results obtained in this study constitute direct evidence that the gene products of each nucleus are uniformly distributed in adult skeletal muscle cells in vivo. Therefore, it appears that the mammalian myofibre is a functional syncytium; multiple myonuclei maintain the cell by way of a common pool of gene products.

VALIDITY OF INTERPRETATION OF RESULTS

1) It could be argued that, between different regions of a muscle fibre, there were small real differences in GPI-1 isozymes which were below the limits of detectability in the quantitative analysis system used. Small differences between sections could arise, for example, if the products of each nucleus were quite widely dispersed but were not uniformly distributed over the whole fibre length. However, because the ispzyme distributions in sections separated by large distances (of the order of 1 mm) did not differ from one another more than the distributions in adjacent sections, it does seem unlikely that there were concentration gradients of the products of each myonucleus along the length of myofibres. For example, in many

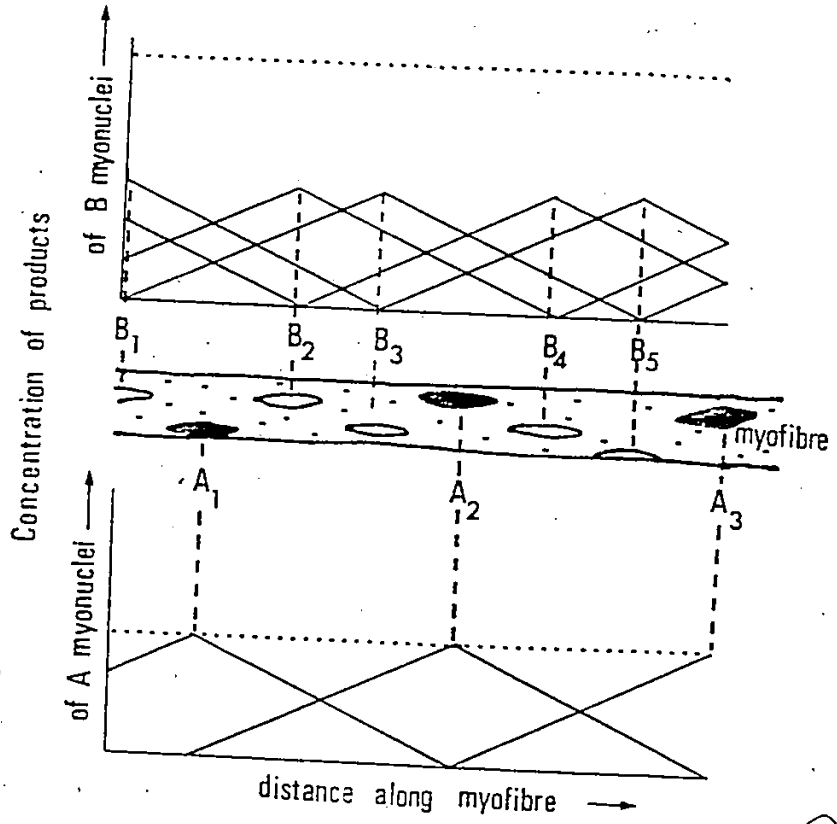
of the mosaic muscle fibres from chimaera No. 145, the proportion of nuclear types was 49 Gpi-1^a/Gpi-1^a:1 Gpi-1^b/Gpi-1^b. If gene products were not completely distributed, one would expect that the proportion of b type monomers would consistently decrease or increase as sections were taken farther away from or closer to the relatively rare regions containing Gpi-1^b nuclei. No such trends were observed.

2) Theoretically, a uniform distribution of isozymes could arise without the gene products of each myonucleus being distributed throughout the entire length of a mosaic cell. If the 2 types of nuclei were arranged in repeating identical sequences along the length of a mosaic fibre, then a homogeneous gene product distribution could result if the products of each nucleus were distributed by diffusion over the length of at least 2 sequences (see Figure 26). The results indicate that such sequences must contain at least 50 nuclei, as the ratio of Gpi-1^a : Gpi-1^b nuclei was 49:1 in some mosaic fibres. All regions of cytoplasm in these cells could have equal access to the products of both types of nuclei if the myonuclei were arranged in repeating sequences of 49 Gpi-1^a nuclei, 1 Gpi-1^b nucleus and if the gene products were distributed over the length of two such sequences, about 1000 µm.

Although the organization of myoblast clones during myogenesis *in vivo* has not been investigated, it appears unlikely that myonuclei from the two types of myoblasts come to be arranged in repeating identical sequences in the myofibres of mouse chimaeras. For example, the probability of randomly selecting the pattern 49 Gpi-1^a, 1 Gpi-1^b, 49 Gpi-1^a, 1 Gpi-1^b, myoblasts from a population which contains myoblasts in the ratio of 49 Gpi-1^a : 1 Gpi-1^b is $2 \times \left(\frac{49}{50}\right)^{49} \times \left(\frac{1}{50}\right) \times \left(\frac{49}{50}\right)^{49} \times \left(\frac{1}{50}\right)$,

Figure 26

Diagram illustrating how uniform distributions of the gene products of 2 types of myonuclei could arise over the entire length of a myofibre when the products of each myonucleus are not distributed throughout the fibre length. It has been assumed that the 2 types of myonuclei are arranged in repeating identical sequences (shown here as 1 A myonucleus followed by 2 B myonuclei). If the products of each myonucleus are distributed along linear concentration gradients (shown by the curves labeled B_1,A_1,B_2,B_3, etc.) that extend over the length of 2 nuclear sequences, then the overall concentration of each type of gene product (indicated by the dotted horizontal lines) will be homogeneous over the entire fibre length.


Concentration of products
of B myonuclei
B1
B2
B3
B4
B5
myofibre
A1
A2
A3
of A myonuclei
distance along myofibre

or approximately .0001.

3) The possibility exists that the regulation of gene expression is different in multinucleated cells which contain genetically different nuclei from that in nonmosaic cells; such a phenomenon could produce differences in functional organization between mosaic and nonmosaic muscle fibres. However, there is no evidence for morphological, biochemical or physiological abnormalities in mosaic myofibres in chimaeras produced from normal mice (Mintz, 1974; Peterson, 1974). In addition, all mammalian females are genetic mosaics as a result of the random inactivation of one or the other X-chromosome early in development (Lyon, 1972). There are no apparent differences between the myofibres of males, which contain genetically identical nuclei, and the fibres of females, which are expected to contain two genetically different types of nuclei. Therefore, it seems likely that the nuclear-cytoplasmic relationship that is described for mosaic myofibres by the results of this study exists also in normal (i.e.) nonmosaic, myofibres.

4) There is no direct evidence that <u>all</u> muscle proteins are maintained by way of a common pool of the gene products of multiple myonuclei. However, the present findings for GPI-1, a cytosolic enzyme, and MOD-2, a protein of the mitochondrial matrix, together with previous reports that heteropolymers of other cytoplasmic enzymes (MOD-1: Mintz and Baker, 1967; Peterson, 1974; Id-1: Baker and Mintz, 1969), are present in muscles from appropriate mouse chimaeras, indicate that the widespread distribution of the precursors of cytoplasmic and organelle-bound proteins may be a common occurrence.

The maintenance of the proteins of the plasma membrane in muscle fibres <u>in vivo</u> has not been studied, but there is evidence from studies of cultured chick myotubes (Fambrough and Devreotes, 1978), that acetyl-

choline receptors (AChR) reach the sarcolemma by way of a direct intracellular membrane pathway from the perinuclear region to the cell surface. However, it is not known whether the synthesis of AChRs at each perinuclear location is directed exclusively by mRNA transcribed in the local nucleus or by mRNA from remote nuclei. Furthermore, even if gene products are transported directly from each myonucleus to the adjacent plasma membrane in mature myofibres in vivo, it is possible that the newly inserted proteins do not remain localized in the adjacent membrane. It is known that membrane proteins, including AChR, are able to diffuse laterally in the fluid matrix of the plasma membrane (Singer and Nicolson, 1972; Edidin and Fambrough, 1973; Anderson and Cohen, 1977).

MECHANISM OF GENE PRODUCT DISTRIBUTION

Except in some pathological conditions, both nuclei and mitochondria are uniformly distributed along the skeletal muscle fibre length, and are thought not to change their relative positions within a mature cell. Therefore, the results obtained in this study indicate that nuclear gene products are able to move about inside the skeletal myofibres of the mouse. It is not clear how the widespread distribution of the products of each nucleus is achieved in myofibres.

In a simple model of diffusion in which a single myonucleus is considered to act as an instantaneous source of a diffusing product, the spreading of the substance along the fibre length can be described by Crank's (1956) equation for diffusion in one dimension along a cylinder of infinite length:

$$C = \frac{M}{2(\pi Dt)^{\frac{1}{2}}} e^{-x^2/4Dt}$$

where C is the concentration of the diffusing substance at a distance x from the source, M is the amount of the substance deposited at time

t=0 at the source x=0, and D is the diffusion coefficient for the substance. According to this model, a diffusing substance such as aequorin (molecular weight 30,000 daltons, D in muscle of $5X10^{-8}$ $cm^2 sec^{-1}$) will be present at x=1mm (i.e. 1 mm on either side of the source) in a concentration that is 1/100 of the concentration at x=0, approximztely 3 hours after the substance begins to diffuse from the source and it will take almost 6 days for the concentration at x=1mm to reach a value that is 9/10 of that at the source (see Table 3). Examples of other proteins of MW approximately 30,000 daltons include thrombin, β-lactoglobulin, and monomers of tropomyosin B. The MW of the murine GPI-1 monomer is reported to be 60,000 (Oster-Granite and Gearhart, 1978) to 62,000 (Charles and Lee, 1980) and murine MOD-2 monomers weigh approximately 62,000 daltons (Bernstine, 1979). In fact, many proteins and RNA species are larger molecules and, therefore might be expected to diffuse more slowly than a 30,000 dalton MW substance. However, as the mathematical relationship between diffusion coefficient and molecular weight has been defined only for small, roughly spherical molecules (Martin et al.,1969), it is not possible to predict exactly how much more slowly the large molecules will spread. The diffusion times may not be much longer as molecular radius may not increase in direct proportion to MW for large globular proteins.

The simple model of diffusion from an instantaneous source is quite clearly not able to accurately describe the diffusion behaviour of a population of macromolecules constantly influenced by synthesis and degradation. However, it does permit the estimation of the minimum times that are required to achieve the widespread distribution of gene products by diffusion.

On the basis of the calculations described above, it appears possible

TABLE 3. APPROXIMATE TIMES FOR DIFFUSION FROM AN INSTANTANEOUS PLANE SOURCE ALONG THE LENGTH OF A CYLINDER*

SUBSTANCE	DIFFUSION COEFFICIENT IN MUSCLE ($cm^2 sec^{-1}$)	REFERENCE	TIME TO REACH C/C_o=.01 AT x CM FROM SOURCE		TIME TO REACH C/C_o=0.9 AT x CM FROM SOURCE	
			x=.1	x=.5	x=.1	x=.5
water	1.4×10^{-5}	Rorschach et al. (1973)	39 sec	16.2 min	29.8 min	12.4 hr
aequorin	5×10^{-8}	Blinks et al. (1978)	3.0 hr	3.1 days	5.8 days	144 days

* The calculations were made by substituting appropriate values in the equations

$$C = \frac{M e^{-x^2/4Dt}}{2\sqrt{\pi Dt}} \quad \text{and} \quad C_o = \frac{M e^{-x_o^2/4Dt}}{2\sqrt{\pi Dt}}$$

and solving for t at given values of C/C_o.

that nuclear products that turn over with half-lives of 10-20 days become approximately uniformly distributed for several millimeters along the length of a myofibre by simple diffusion. Many sarcoplasmic and myo-fibrillar proteins have half-lives in this range (see Introduction). However, some muscle proteins turn over more rapidly. Dolken and Pette (1974) have reported half-lives on the order of one day for several glycolytic enzymes in rabbit muscle. Therefore, the concentration of the products of individual myonuclei cannot approach uniformity over appreciable fibre lengths by simple diffusion if the products turn over at such high rates. One possibility is that the gene product that is actually distributed has a half-life that is longer than that of the final product and, therefore, simple diffusion is an adequate mechanism for distribution. For example, relatively stable mRNAs and/or protein precursors may exist. Another possibility is that transport processes other than diffusion (for example, the mechanical mixing of the soluble contents during myofibre contraction) carry out the rapid intracellular distribution of gene products. At present there is no direct evidence for or against either possibility.

Two observations indicate that the widespread distribution of gene products in myofibres does not depend on properties peculiar to adult, innervated cells:1) In a preliminary study, I found that GPI-1 heteropolymers persisted in fibres denervated for 21 days. Denervation of muscle leads to marked atrophy(e.g., Tower 1939). During denervation atrophy, myofibres lose their well organized ultrastructural appearance (e.g. Hudgson and Mastaglia, 1974) and it is known that the rates, and perhaps the sites, of protein synthesis and degradation are changed (Goldberg, 1969; Gauthier and Schaeffer, 1974;Millward, 1976). In spite of the alterations in cell structure and function, the products of multiple myonuclei can

interact in denervated muscle fibres.

2) Heteropolymers of GPI-1 were found in muscle from embryonic chimaeras at day 16 *in utero* (Cross, unpublished results). Myoblasts which are mononucleated and therefore contain no heteropolymers, are incorporated into myotubes over days 13½ to 15 *in utero* in the mouse (Pai, 1965). Therefore, the finding of relatively large heteropolymer bands in muscles at embryonic day 16 indicates that there is extensive and rapid interaction between the gene products of multiple nuclei in young myofibres. The ultrastructure and functional organization of these young myofibres differ in many respects from mature myofibres (see Introduction). However, the 'adult' organization with respect to the biosynthesis of at least one gene product (GPI-1) appears to be established very soon after myoblast fusion.

In view of the observations on denervated and young myofibres, it appears unlikely that a highly specialized transport mechanism is required for the distribution of the products of individual myonuclei throughout a muscle fibre.

NATURE OF GENE PRODUCTS DISTRIBUTED

The results obtained in this study clearly indicate that individual myonuclei do not contribute gene products directly and exclusively to the nearest sites of enzyme assembly and localization. Rather, muscle proteins can be assembled from gene products encoded by multiple, genetically different myonuclei. Theoretically, there are three general possibilities regarding the level of protein biosynthesis at which the widespread distribution of gene products occurs.

Messenger RNA

If mRNAs become widely dispersed from the myonuclei in which they were transcribed, then homologous mRNAs encoded in different nuclei may be translated at common sites and homologous protein monomers encoded

by different nuclei may interact in the formation of a final oligomeric product.

If, as suggested by Shore and Tata (1977), particular proteins are synthesized close to the site of their action, then the present results can be explained only if mRNAs become homogeneously dispersed throughout the cytoplasm of muscle cells before they bind to specific translation sites. For example, if monomers of nuclear-coded mitochondrial proteins are synthesized on ribosomes associated with the mitochondrial outer membrane and discharged directly into mitochondria (i.e. vectorial translation; e.g. Kellems, Allison and Butow, 1974), then the present results indicate that single mitochondria have access to mRNAs from multiple myonuclei.

The half-lives of some mRNA species, notably the message for the myosin heavy chain, increase during myogenesis in vitro (Yaffe & Feldman, 1964; Buckingham et al., 1977; Strohman et al., 1977) but very little is known about the turnover of specific mRNAs in mature muscle in vivo. The half-life for ribosomal RNA in rabbit muscle was estimated at 11.5 days (Erdos and Bessada, 1966) and total RNA in rat muscle has a half-life of 17 days (Gerber, Gerber and Altman, 1960). In mature skeletal muscle, total RNA synthesis is low (Yaffe & Fuchs, 1967), RNA polymerase activity is low (Marchok, 1972; van der Westhuyzen, 1979), and there appears to be little ribonuclease (Roth and Milstein, 1952). Therefore, it is possible that most mRNA species are long-lived in muscle. According to the diffusion kinetics described above, there may be enough time for the mRNAs transcribed in different myonuclei to become homogeneously distributed by diffusion.

At present, there is no direct evidence for or against the hypothesis

that the mRNAs are the products of the Gpi-1 and Mod-2 genes that are widely distributed in muscle cells. The specific mRNAs have not yet been identified and the intracellular sites of monomer and oligomer synthesis remain unknown for both proteins.

Monomers

Translation products synthesized on membrane-free ribosomes (Nihei, 1971), or possibly released into the cytoplasm after synthesis on membrane-bound ribosomes, may become part of a large intracellular pool of protein precursors so that monomers encoded by many different nuclei become available at sites of oligomer assembly and at sites where proteins are incorporated into specific intracellular locations. There is evidence that the precursors of monomers, or the monomers themselves, of some oligomeric proteins are free in the cytoplasm of cells (e.g. β-glucuronidase, cytochrome oxidase; see Wickner, 1979, for references). In addition, Webster, Patel, Freeman and Papahadjopoulos (1979) have presented indirect evidence that monomers of malate dehydrogenase are taken up into mitochondria from the cytoplasm. These observations indicate that the process of monomer synthesis and oligomer formation do not necessarily occur in temporal and spatial proximity within cells.

Two lines of evidence indicate that GPI-1 monomers do not exist freely in the cytoplasm of muscle cells:

1) When $Gpi\text{-}1^{a}/Gpi\text{-}1^{a}$ and $Gpi\text{-}1^{b}/Gpi\text{-}1^{b}$ muscles are homogenized together and the homogenates are concentrated, no GPI-1AB dimers are generated (these results). There is evidence that monomer: dimer ratios of some cytoplasmic proteins are concentration dependent, with dimer formation favoured at higher concentrations (Kleile, Schulz, Harrison and Gregory, 1977). Therefore, if free monomers of GPI-1 are present in muscle cells, AB dimers would likely be formed when mixed muscle samples

are concentrated.

2) Free monomers obtained by the in vitro dissociation of purified GPI-1 dimers re-aggregate spontaneously under most conditions (Carter and Yoshida, 1969). Therefore, it is unlikely that the intracellular conditions allow free GPI-1 monomers to exist.

It also appears that there are no free monomers of MOD-2 present in muscle; MOD-2 heteropolymers are not present in mixed homogenates of $Mod\text{-}2^a/Mod\text{-}2^b$ and $Mod\text{-}2^b/Mod\text{-}2^b$ muscles (these results).

Therefore, it is unlikely that the enzyme monomers themselves are the products of the Gpi-1 and $Mod\text{-}2^a$ genes that are widely distributed in skeletal muscle cells. However, it is possible that both genes code for stable monomer precursors that are extensively dispersed before post-translational modifications produce monomers that are able to assemble into oligomers. For MOD-2, post-translational processing may also enable MOD-2 monomers to become incorporated into mitochondria. At present, it is not known if protein precursors of the GPI-1 and MOD-2 monomers are produced in any cells.

Oligomers

It is possible that oligomers are initially assembled in territories, each associated with a single myonucleus, but subsequently are widely distributed; the results obtained in this study could be explained if the process of oligomer formation is reversible and if monomers are re-used during random re-aggregation at sites that have had access to all oligomer types. The assembly of several oligomeric proteins is known to be reversible. For example, the regulation of both the enzyme phosphofructokinase (Mansour, 1972; Friedman and Beychok, 1979) and the structural protein tubulin (Dunne and Wood, 1975) may depend on the sensitivity of oligomeric dissociation and re-association to cellular conditions, and similar controlled equilibria between the assembly and dissociation of oligomeric

proteins may exist in muscle cells. However, there is also evidence that the assembly of some cytoplasmic oligomeric enzymes is irreversible. Goldstein and Gartler (1979) have presented convincing evidence that the association of monomers to form glucose-6-phosphate dehydrogenase is irreversible in human-mouse fibroblast heterokaryons. In addition, Bleile et al., (1977) have shown that cytoplasmic malate dehydrogenase does not dissociate into monomers, even under conditions of extreme dilution and low pH.

Evidence that GPI-1 monomers are not re-cycled comes from studies of $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^c}$ heterozygous mice (Peterson, unpublished results). The $\underline{Gpi\text{-}1^c}$ allele codes for monomers which, when assembled into CC dimers, appear to be rapidly degraded. Red blood cells of $\underline{Gpi\text{-}1^c}/\underline{Gpi\text{-}1^c}$ mice are deficient in GPI-1 activity. The deficiency is thought to occur because the GPI-1C which is degraded is not replaced by protein synthesis (Padua et al., 1978). In red blood cells of $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^c}$ mice, one might expect that if the processes of dimer dissociation and random re-aggregation of monomers occur during the selective degradation of GPI-1C dimers, eventually both the GPI-1C and GPI-1AC bands would be lost from the cells. The C monomers remaining in AC dimers would regenerate some CC dimers during each cycle of dissociation and re-association. In their turn, each group of regenerated CC dimers would be destroyed until virtually no C monomers remained for the assembly of either CC or AC dimers. In fact, the ratio of GPI-1C : GPI-1AC : GPI-1A is 0:2:1 in red blood cells from mature $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^c}$ mice. This is the result expected if the assembly of GPI-1 dimers is an irreversible process; the C monomers that were initially incorporated into AC dimers persist in the stable GPI-1AC isozyme band.

Further evidence that GPI-1 dimers do not dissociate and re-assemble is the observation that GPI-1 heteropolymers are not generated *in vitro* when homogenates of *Gpi-1*^{a}/*Gpi-1*^{a} and *Gpi-1*^{b}/*Gpi-1*^{b} muscles are incubated together (these results).

In view of the indications that GPI-1 dimer formation is not a reversible process, it appears unlikely that GPI-1 heteropolymers arise in mosaic myofibres by the widespread distribution of and exchange of monomers between previously assembled homopolymers.

It is also unlikely that oligomers of MOD-2 are assembled prior to their widespread distribution in the cytoplasm of muscle cells; several observations indicate that MOD-2 oligomers may not exist at all in the cytoplasm. For example, Bernstine (1979) reported that there is no MOD-2 activity in the post-mitochondrial supernatant of mouse kidney homogenates. It is also known that the oligomers of another mitochondrial enzyme, malate dehydrogenase, are dissociated under conditions such as enzyme dilution and low substrate or coenzyme concentration (Bleile *et al.*, 1977). Thus, conditions in the cytoplasm of muscle fibres may inhibit the assembly of MOD-2 oligomers. The formation of oligomers may occur only after the monomers or monomer precursors are incorporated into mitochondria.

In summary, it appears most likely that the widespread distribution of the *Gpi-1* and *Mod-2* gene products in skeletal muscle cells occurs at the level of the mRNAs and/or protein precursors of the enzyme monomers.

RELEVANCE OF PRESENT FINDINGS TO OTHER QUESTIONS

The finding that multiple nuclei cooperate in the maintenance of cytoplasmic and mitochondrial-bound proteins in multinucleated myofibres will be of interest to investigators of a number of problems in muscle biology.

Carriers of X-linked Muscle Diseases

Although it remains to be determined whether the myonucleus is the primary site of expression of the abnormal gene for any of the X-linked human muscle diseases, the results obtained in this study suggest that mosaic myofibres in carriers of such myopathies could become intermediately affected over their entire length as a result of the homogeneous distribution of the normal and abnormal gene products.

Trophic Mechanisms

The present results indicate that the neurotrophic mechanisms must have a complex organization. On the basis of the findings presented here, it is expected that a neurotrophic agent that enters a myofibre at the neuromuscular junction may have access to myonuclei everywhere in the fibre and it is also expected that the products of myonuclei affected by the trophic substance will be distributed throughout the myofibre. The neural control of muscle properties that are expressed throughout the length of a muscle fibre (for example, the maintenance of an appropriate resting membrane potential) may well be mediated by such a diffuse mechanism. However, the expression of some neurotrophic effects (for example, the induction of high density packing of acetylcholine receptors) is localized at the region of the neuromuscular junctional. Additional mechanisms must be involved in the specific localization of such effects.

Number of Functional Mybnuclei

Several investigators have reported that there is a direct and constant relationship between the number of myonuclei and the volume of cytoplasm in mature myofibres and have suggested that the growth potential of a muscle is limited by the number of nuclei it contains (e.g., Burleigh, 1977; Kelly, 1978). Implicit in this suggestion is the assumption that all the myonuclei are functional; However, no previous studies have

addressed the question of how many nuclei are active in directing the synthesis of mRNA within a myofibre.

In the present study, it was possible to determine, for muscle cells that contained two genetically different types of myonuclei, the ratio of the two nuclear types that was active within a single cell. In mosaic myofibres from $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^a} \leftrightarrow \underline{Gpi\text{-}1^b}/\underline{Gpi\text{-}1^b}$ mouse chimaeras, both Gpi-1 alleles are expected to be equally active in directing the synthesis of GPI-1 monomers (see Introduction). Therefore, the observation that the ratio of A:B GPI-1 monomers was 49:1 in some myofibres (see Results) indicates that the ratio of nuclei directing GPI-1 synthesis was also 49 $\underline{Gpi\text{-}1^a}/\underline{Gpi\text{-}1^a}$: 1 $\underline{Gpi\text{-}1^b}/\underline{Gpi\text{-}1^b}$; at least 50 nuclei were functional within these single myofibres and the actual number of active myonuclei could have been any multiple of 50.

The fact that, within a mosaic muscle, the distribution of MOD-2 isozymes paralleled that of the GPI-1 isozymes (see Results, Table 2) constitutes indirect evidence that the myonuclei that are active in directing the synthesis of GPI-1 are also responsible for the synthesis of MOD-2. Therefore, there was no indication that the synthesis of different gene products is specified by different groups of myonuclei.

The present results are consistent with the hypothesis that the population of active myonuclei is large and may, in fact, contain all the nuclei within a muscle fibre.

APPENDICES

APPENDIX 1A. DISTRIBUTIONS OF GPI-1 ISOZYMES IN WHOLE MUSCLE CROSS-SECTIONS FROM HETEROZYGOUS CONTROL MICE.

SECTION THICKNESS 20 µm.

SECTION NUMBER	%GPI-1		
	A	AB	B
3	26.7	52.7	20.6
4	26.8	52.1	21.1
5	25.1	52.1	22.8
6	26.6	52.0	21.4
7	26.0	54.2	19.8
8	32.1	50.6	17.3
17	25.7	49.2	25.1
18	27.9	48.5	23.6
21	26.5	52.3	21.2
22	28.8	50.6	20.6
23	23.8	50.4	25.8
24	22.1	55.8	22.1
25	29.5	50.0	20.5
26	33.3	47.6	19.1
27	35.6	47.4	17.0
28	31.2	48.4	20.4
29	26.8	49.9	23.3
30	34.3	48.5	17.2
31	32.4	49.7	17.9
32	31.0	50.6	18.4
35	30.3	49.4	20.3
36	29.6	50.5	19.9
37	35.0	45.6	19.4
38	30.3	49.7	20.0
39	35.7	46.0	18.3
40	29.8	48.6	21.6
41	32.1	49.1	18.8
42	35.5	46.8	17.7
43	30.4	46.5	23.1
44	32.7	47.2	20.1
45	34.8	46.0	19.2
46	36.5	46.1	17.4
47	30.7	42.4	26.9
48	36.2	44.4	19.4
49	34.3	46.6	19.1
50	30.5	49.8	19.7
51	30.8	51.1	18.1
52	32.6	45.9	21.5
53	35.0	45.6	19.4
54	34.6	45.8	18.3
55	33.0	47.2	19.8

APPENDIX 1A (cont'd).

56	32.3	48.4	19.3
57	29.0	47.1	23.9
58	29.8	48.6	21.6

SECTION THICKNESS 30 µm.

SECTION NUMBER	% GPI-1		
	A	AB	B
1	38.7	46.7	14.6
2	33.1	49.3	17.6
3	36.3	48.1	15.6
4	33.7	52.9	13.4
5	32.5	51.9	15.6
6	33.4	50.7	15.9
7	38.1	45.7	16.2
8	36.3	47.4	16.3
9	33.5	47.1	19.4
10	36.1	46.2	17.7
13	35.1	48.1	16.8
14	37.9	45.5	16.6
15	37.6	45.8	16.6
16	41.6	44.3	14.1
17	35.6	48.3	16.1
18	40.4	46.2	13.4
19	40.4	45.9	13.7
20	34.1	47.7	18.2
21	39.9	45.2	14.9
22	37.4	46.0	16.6
23	41.9	46.0	12.1
24	39.0	46.0	15.0
25	34.4	48.9	16.7
26	36.4	49.2	14.4
27	35.5	46.2	18.3
28	32.7	46.3	21.0
31	37.7	48.9	13.4
32	37.0	47.8	15.2
33	29.9	44.8	25.3
34	35.4	47.1	17.5
35	37.7	49.6	12.7
36	32.2	47.8	20.0
37	37.1	46.3	16.6
38	39.5	45.4	15.1
41	35.8	47.7	16.5
42	40.7	46.1	13.2

APPENDIX 1A (cont'd).

43	35.6	46.6	17.8
44	38.2	47.8	14.0
45	28.5	47.1	24.4
46	34.3	47.3	18.4
49	35.2	45.2	19.6
50	35.9	50.7	13.4
51	28.9	51.2	19.9
52	39.9	47.5	12.6

SECTION THICKNESS 40 µm.

SECTION NUMBER	% GPI-1		
	A	AB	B
1	28.9	47.3	23.8
2	31.2	45.4	23.4
3	33.9	44.3	21.8
4	31.9	46.9	21.2
5	27.8	46.4	25.8
6	31.0	43.6	25.4
7	30.9	43.3	25.8
8	31.3	45.2	23.5
9	35.1	45.0	19.9
10	34.1	44.3	21.6
11	30.1	41.9	28.0
12	33.4	43.4	23.2
13	37.1	40.3	22.6
14	35.4	41.3	23.3
15	37.9	40.1	22.0
16	38.5	39.0	22.5
17	39.3	40.8	19.9
18	36.2	41.6	22.2
19	34.2	41.0	24.8
20	39.6	39.9	20.5
21	39.2	41.6	19.2
22	36.0	41.7	22.3
25	38.2	41.2	20.6
26	37.3	41.6	21.1
27	34.2	44.5	21.3
28	34.2	41.9	23.9
29	32.3	41.4	26.3
30	35.5	44.7	19.8
31	34.9	37.5	27.6
32	35.3	41.8	22.9
33	33.5	41.6	24.9
34	34.4	41.3	24.3

APPENDIX 1A (cont'd).

37			
38	33.9	40.6	25.5
39	32.6	41.2	26.2
40	35.5	46.8	17.7
41	34.6	41.5	23.9
42	34.6	42.4	23.0
43	37.2	41.2	21.6
44	35.8	40.8	23.4
45	35.9	41.8	22.3
46	36.9	41.3	21.8
47	38.5	42.1	19.4
48	35.1	40.9	24.0
49	34.3	40.6	25.1
50	36.9	44.5	18.6
51	35.8	42.6	21.6
52	32.4	40.8	26.8
	38.2	37.6	24.2

APPENDIX 1B. DISTRIBUTION OF GPI-1 ISOZYMES IN CROSS-SECTIONS OF SINGLE MUSCLE FIBRES FROM HETEROZYGOUS CONTROL MICE.

SECTION THICKNESS 20 µm.

FIBRE NO.	SECTION NO.	% GPI-1		
		A	AB	B
1	1	18.1	48.6	33.3
	2	27.7	52.1	20.2
	3	18.5	56.7	24.8
2	1	19.9	52.2	27.9
	2	20.4	52.5	27.1
	3	23.5	52.3	24.2
3	1	28.2	54.6	17.2
	2	29.1	51.4	19.5
4	1	24.1	48.2	27.7
	2	27.2	49.7	23.1
5	1	20.3	47.7	32.0
	2	21.1	54.4	24.5
	3	25.6	58.7	15.7
6	1	25.7	44.1	30.2
	3	19.2	59.9	20.9
7	1	26.2	52.2	21.6
	2	27.1	52.1	20.8
	3	21.5	56.0	22.5
8	1	29.0	50.2	20.8
	2	23.5	57.1	19.4
	3	24.0	53.8	22.2
9	1	31.8	50.1	18.1
	2	21.8	54.6	23.6
	3	22.0	55.2	22.8
10	1	31.1	47.2	21.7
	2	21.3	57.5	21.2
	3	19.7	58.6	21.7
11	1	29.4	49.7	20.9
	2	17.1	57.9	25.0
	3	16.8	56.4	26.8
12	1	32.2	45.8	22.0
	2	21.9	57.9	20.2
	3	20.0	56.4	23.6
13	1	21.7	52.5	25.8
	3	22.9	51.8	25.3
14	1	24.6	50.8	24.6
	2	19.5	59.3	21.2
	3	20.6	50.4	29.0
15	1	27.6	46.9	25.5
	3	24.4	57.8	17.8

APPENDIX 1B (cont'd).

16	1	22.3	52.7	25.0
	2	11.1	57.5	31.4
	3	23.1	55.8	21.1
17	1	15.3	57.5	27.7
	2	24.0	53.4	22.6
	3	16.7	57.3	26.0
18	1	21.1	56.2	22.7
	2	24.1	54.5	21.4
	3	25.9	51.2	22.9
19	1	22.2	55.9	21.9
	2	19.8	50.8	29.4
	3	20.3	53.8	25.9
20	1	17.4	62.2	20.4
	2	21.5	53.8	24.7
	3	21.9	55.1	23.0
21	1	21.8	55.6	22.6
	2	24.6	55.6	19.8
22	1	25.1	50.6	24.3
	2	20.4	54.3	25.3
	3	25.7	54.1	20.2
23	1	24.2	52.7	23.1
	2	23.7	54.2	22.1
24	1	24.7	53.5	21.8
	2	20.5	50.2	29.3
25	1	22.4	54.9	22.7
	3	16.0	53.1	30.9
26	1	21.2	53.6	25.2
	2	23.9	53.0	23.1
	3	19.9	53.9	26.2
27	1	14.7	57.9	27.4
	2	24.8	51.9	23.3
	3	19.3	50.7	30.0
28	1	29.9	53.2	16.9
	2	27.1	53.1	19.8
29	1	30.0	54.4	15.6
	2	32.0	51.1	16.9
30	1	23.8	56.3	19.9
	2	29.2	52.8	18.0
31	1	20.4	55.2	24.4
	2	20.5	45.9	33.6
32	1	22.6	50.2	27.2
	2	26.4	47.9	25.7
33	1	26.5	51.4	22.1
	2	25.4	53.1	21.5
34	1	23.7	53.6	22.7
	2	26.7	50.1	23.2
35	1	26.1	52.2	21.7
	2	22.6	45.8	31.6

APPENDIX 1B (cont'd).

35	1	26.1	52.2	21.7
	2	22.6	45.8	31.6
36	1	24.5	53.5	22.0
	2	19.6	50.8	29.6
37	1	19.0	52.6	28.4
	2	27.5	53.2	19.3
38	1	19.4	52.2	28.4
	2	25.9	52.1	22.0
39	1	26.1	52.9	21.0
	2	21.9	54.4	23.7
40	1	21.9	54.6	23.5
	2	23.1	53.6	23.3

SECTION THICKNESS 30 µm.

FIBRE NO.	SECTION NO.	% GPI-1		
		A	AB	B
4	1	26.0	55.5	18.5
	2	20.5	56.3	23.2
5	1	19.4	48.9	31.7
	2	21.3	54.7	24.0
6	1	21.1	53.9	25.0
	2	24.1	52.7	23.2
7	1	22.5	54.0	23.5
	2	21.4	54.9	23.7
10	1	31.0	48.3	20.7
	2	24.8	52.8	22.4
11	1	25.9	52.6	21.5
	2	26.3	52.2	21.5
12	1	26.6	56.0	17.4
	2	19.9	59.4	20.7
13	1	26.2	48.3	25.5
	2	26.1	52.8	21.1
16	1	29.6	47.7	22.7
	2	23.2	55.9	20.9
17	1	21.5	53.9	24.6
	2	20.3	54.7	25.0
18	1	18.4	54.0	27.6
	2	20.9	56.3	22.8
19	1	23.7	53.2	23.1
	2	20.8	61.2	18.0
23	1	17.5	57.8	24.7
	2	19.1	57.0	23.9
24	1	21.1	54.1	24.8
	2	24.0	51.4	24.6

APPENDIX 1B (cont'd).

SECTION THICKNESS 40 µm.

FIBRE NO.	SECTION NO.	% GPI-1		
		A	AB	B
1	1	33.3	45.2	21.5
	2	25.9	53.6	20.5
2	1	26.2	49.7	24.1
	2	24.3	50.3	25.4
3	1	28.1	49.4	22.5
	2	22.0	52.3	25.7
4	1	28.4	51.2	20.4
	2	24.2	51.7	24.1
5	1	23.6	50.0	26.4
	2	24.7	49.6	25.7
6	1	25.2	51.5	23.3
	2	26.9	47.5	25.6
9	1	23.5	50.4	26.1
	2	21.6	52.1	26.3
10	1	23.1	56.4	20.5
	2	25.1	49.7	25.2
11	1	25.1	50.8	24.1
	2	25.2	50.2	24.6
13	1	20.7	51.2	28.1
	2	28.5	48.8	22.7
14	1	23.2	50.7	26.1
	2	20.4	53.6	26.0
15	1	20.9	50.7	28.4
	2	16.5	52.3	31.2
16	1	18.3	57.9	23.8
	2	19.6	55.6	24.8
19	1	26.0	51.4	22.6
	2	28.2	52.6	19.2
20	1	19.9	62.4	17.7
	2	21.8	53.6	24.6
21	1	20.8	58.9	20.3
	2	28.0	52.1	19.9
22	1	23.4	47.9	28.7
	2	12.8	58.7	28.5
23	1	25.3	49.2	25.5
	2	22.9	57.7	19.4
24	1	26.0	49.3	24.7
	2	29.1	49.9	21.0

APPENDIX 2. RELATIVE PROPORTION OF GPI-1AB IN CONTROL SAMPLES FROM HETEROZYGOUS MICE

% GPI-1AB	
MEASURED	PREDICTED
56.1	49.8
56.7	49.3
56.1	49.2
60.8	49.9
55.9	49.9
59.3	49.8
54.4	49.9
43.7	50.0
44.3	49.9
42.4	49.8
41.8	50.0
60.1	49.9
45.8	49.9
48.9	50.0
45.7	49.8
47.6	49.5
41.9	49.8
40.7	49.8
39.0	49.7
42.7	50.0
36.8	50.0
53.2	49.9
56.8	49.8
55.1	50.0
52.2	50.0
51.5	50.0
50.5	49.8
49.7	49.6
49.7	49.8
45.1	50.0
47.9	49.3
27.7	49.9
44.3	49.8

APPENDIX 3A. DISTRIBUTIONS OF GPI-1 ISOZYMES IN CROSS-SECTIONS OF WHOLE MOSAIC MUSCLES.

CHIMAERA 32, LEFT EDL, SECTION THICKNESS 20 µm.

SECTION NO.	% GPI-1		
	A	AB	B
1-5	91.6	4.3	3.1
11-15	93.9	4.1	2.0
41-45	94.7	4.9	0.4
101-115	93.5	6.1	1.4

CHIMAERA 32, RIGHT ECRL, SECTION THICKNESS 10 µm.

SECTION NO.	% GPI-1		
	A	AB	B
1-10	97.6	2.0	0.4
21-30	94.2	4.2	1.6
46-50	94.5	4.6	0.9
51-55	94.8	4.5	0.7
76-80	87.2	9.8	3.0
81-85	90.0	6.9	3.1
96-100	93.3	6.0	0.7
106-110	90.7	8.3	1.0
126-130	94.6	4.4	1.0
166-170	88.3	8.1	3.6
171-175	90.7	7.4	1.9
176-180	89.6	7.7	2.7
181-185	90.5	7.9	1.6
191-195	88.5	9.7	1.8
196-205	91.6	6.0	2.4

CHIMAERA 38, RIGHT AT, SECTION THICKNESS 10 µm.

SECTION NO.	% GPI-1		
	A	AB	B
1-25	39.7	35.8	24.5
26-30	37.2	39.1	23.7
81-90	38.4	38.2	23.4
196-205	33.0	40.5	26.5

APPENDIX 3A (cont'd).

261-265	39.6	37.6	22.8
316-325	34.2	38.3	27.5

CHIMAERA 38, LEFT VASTUS LATERALIS, SECTION THICKNESS 10 µm.

SECTION NO.	% GPI-1		
	A	AB	B
1-25	39.8	38.2	22.0
26-30	34.3	42.6	23.1
81-90	37.1	36.8	26.1
141-145	42.1	36.8	21.1
196-205	37.6	37.2	25.2

CHIMAERA 189, RIGHT AT, SECTION THICKNESS 30 µm.

SECTION NO.	% GPI-1		
	A	AB	B
1	20.6	36.6	42.8
2	20.9	39.0	40.1
3	28.6	36.6	34.8
4	28.0	32.7	39.3
5	23.9	37.6	38.5
6	28.4	38.5	33.1
7	23.2	39.3	37.5
8	25.5	37.1	37.4
9	26.0	37.2	36.8
10	25.6	38.0	36.4
11	30.1	34.1	35.8
12	31.0	35.8	33.2

APPENDIX 3B. DISTRIBUTION OF GPI-1 ISOZYMES IN SINGLE MUSCLE FIBRES FROM MOUSE CHIMAERAS.

CROSS-SECTIONS, 20 µm.

CHIMAERA 115, LEFT AT

FIBRE NO.	SECTION NO.	% GPI-1		
		A	AB	B
1	1	11.7	39.8	48.5
	4	9.3	41.7	49.0
2	1	33.4	48.7	17.9
	3	33.9	47.5	18.6
	4	39.5	43.7	16.8
	5	34.4	47.7	17.9
3	1	31.7	41.1	27.2
	3	26.5	46.5	27.0
	4	31.9	45.4	22.7
5	1	32.0	48.7	19.3
	3	31.2	47.9	20.9
	4	36.7	47.2	16.1
6	1	28.2	49.3	22.5
	3	36.5	49.9	13.6
	4	29.5	49.9	20.2
7	1	11.3	49.2	39.5
	3	18.0	47.0	35.0
	4	15.0	47.3	37.7
8	1	21.6	49.5	28.9
	3	20.2	50.0	29.8
9	1	32.1	47.5	20.4
	3	38.7	45.4	15.9
	4	38.7	42.3	19.0
10	1	28.3	49.1	22.6
	3	22.2	53.2	24.6
	4	29.5	51.4	19.1
12	1	17.8	49.6	32.6
	3	17.5	47.0	35.5
	4	11.5	45.8	42.7

CHIMAERA 142, RIGHT AT

FIBRE NO.	SECTION NO.	% GPI-1		
		A	AB	B
1	1	46.3	43.0	10.7
	2	50.5	40.0	9.5

APPENDIX 3B (cont'd).

	4	59.5	35.2	5.3
2	1	27.7	34.9	37.4
	2	20.8	43.0	36.2
	4	13.7	49.5	36.8
3	1	47.1	42.6	10.3
	2	51.5	39.8	8.7
	4	55.0	40.6	4.4
4	1	46.3	44.1	9.6
	2	47.9	41.0	11.1
	4	55.7	38.4	5.9
6	1	39.0	49.3	11.7
	2	40.8	46.3	12.9
	4	46.0	41.5	12.5
7	1	32.0	46.1	21.9
	2	29.7	45.2	25.1
	4	45.6	41.4	13.0
8	2	15.1	45.4	39.5
	4	7.9	41.3	50.8
9	1	31.7	40.7	27.6
	2	42.9	35.4	21.7
	4	36.7	46.7	16.6

CHIMAERA 145, LEFT AT

FIBRE NO.	SECTION NO.	A	AB	B
1	4	96.2	3.8	n.d.*
	7	94.6	5.4	n.d.
	12	94.8	5.2	n.d.
	16	97.4	2.6	n.d.
2	4	97.8	2.2	n.d.
	7	94.5	5.5	n.d.
	12	97.5	2.5	n.d.
	16	94.7	5.3	n.d.
3	4	90.8	9.2	n.d.
	7	97.6	2.4	n.d.
	12	98.0	2.0	n.d.
	16	97.0	3.0	n.d.
	22	95.5	4.5	n.d.
4	4	95.1	4.9	n.d.
	12	94.7	5.3	n.d.
	16	95.8	4.2	n.d.
	22	94.1	5.9	n.d.
5	4	95.1	4.9	n.d.
	7	93.7	6.3	n.d.

*n.d.: none detectable

APPENDIX 3B (cont'd).

	16	94.8	5.2	n.d.
	22	98.2	1.8	n.d.
6	7	93.4	6.6	n.d.
	12	94.9	5.1	n.d.
	16	92.9	7.1	n.d.
	22	97.6	2.4	n.d.
7	7	96.6	3.4	n.d.
	12	94.6	5.4	n.d.
	16	91.1	8.9	n.d.
8	12	93.8	6.2	n.d.
	22	96.1	3.9	n.d.
9	1	88.7	11.3	n.d.
	7	90.1	9.9	n.d.
	16	96.2	3.8	n.d.
	22	93.8	6.2	n.d.
10	1	98.1	1.9	n.d.
	7	92.6	7.4	n.d.
	22	94.2	5.8	n.d.
11	1	95.1	4.9	n.d.
	4	98.8	1.2	n.d.
	7	93.2	6.8	n.d.
	12	96.5	3.5	n.d.
	16	96.0	4.0	n.d.
	22	95.7	4.3	n.d.
12	1	93.3	6.7	n.d.
	4	95.6	4.4	n.d.
	12	92.5	7.5	n.d.
	16	96.1	3.9	n.d.
	22	97.7	2.3	n.d.
13	4	91.9	8.1	n.d.
	12	94.5	5.5	n.d.
	16	94.5	5.5	n.d.
	22	94.6	5.4	n.d.
14	4	96.1	3.9	n.d.
	7	92.9	7.1	n.d.
	16	90.8	9.2	n.d.

CHIMAERA 149, RIGHT AT

FIBRE NO.(SDH) SECTION NO.

		A	AB	B
1(low)	1	44.5	41.8	13.7
	8	44.4	45.9	9.7
	15	42.6	47.5	9.9
	19	41.8	45.7	12.5
	48	52.6	42.5	4.9
	51	53.0	42.3	4.7

APPENDIX 3B (cont'd).

2 (int)	8	47.7	41.5	10.8
	15	49.1	45.4	5.5
	29	39.6	51.9	8.5
	34	44.4	45.7	9.9
	41	50.7	42.7	6.6
	51	53.2	42.9	3.9
3A (int)	8	45.5	47.2	7.3
	15	44.7	45.5	9.8
	19	43.8	47.4	8.8
	25	40.1	43.4	16.5
	34	52.5	41.5	6.0
	41	53.7	39.3	7.0
	48	50.1	42.6	7.3
	51	50.4	42.0	7.6
3B (int)	25	47.4	42.8	9.8
	29	53.3	40.9	5.8
	34	54.3	39.6	6.1
4 (low)	8	50.0	39.9	10.0
	25	57.1	30.1	12.8
	29	51.0	40.3	7.9
	41	48.5	45.0	8.7
	51	51.4	40.1	6.5
5 (low)	15	48.0	42.6	9.4
	19	44.2	40.6	15.2
	34	45.8	46.3	8.4
	41	52.3	43.4	4.3
	48	53.0	42.5	4.5
	51	62.5	32.5	5.0
6 (high)	41	48.4	44.7	6.9
	48	53.3	42.9	3.8
	51	62.5	32.4	5.1
7 (high)	19	49.7	40.5	9.8
	34	50.1	35.6	14.3
	41	48.5	39.9	11.6
	48	48.9	45.4	5.7
	51	47.8	45.4	6.8
8 (low)	15	47.4	41.9	10.7
	25	47.9	36.4	15.7
	34	44.6	43.3	12.1
	41	50.3	45.5	4.2
	51	47.5	41.1	11.4
9 (int)	8	47.8	42.7	9.5
	15	47.4	45.5	7.1
	25	48.4	40.2	11.4
	29	51.4	41.8	6.8
	34	56.5	39.1	4.0
	41	52.3	42.7	5.0
	51	62.9	31.5	5.6

APPENDIX 3B (cont'd).

10 (low)	1	49.0	44.1	6.9
	8	54.2	40.6	5.2
	15	47.9	44.5	7.6
	34	56.3	39.0	4.7
	41	51.5	44.0	4.5
11 (int)	1	50.5	42.9	6.6
	8	47.5	43.8	8.7
	15	55.1	41.9	3.0
	25	55.7	39.6	4.7
	29	49.0	46.5	4.5
	34	56.4	38.7	4.9
	41	55.4	39.8	4.8
	48	53.6	43.8	2.6
	51	58.1	35.7	6.2
12 (low)	25	49.4	43.3	7.3
	41	52.1	41.4	6.5
	48	50.4	45.6	4.0
	51	53.9	40.5	5.6
13 (high)	19	56.5	38.2	5.3
	41	64.4	29.1	6.5
	48	61.2	30.6	8.2
	51	56.5	38.9	4.6
14 (low)	1	37.2	49.6	13.2
	25	52.5	42.6	4.9
	41	47.0	41.0	12.0
	51	40.0	45.8	14.2
15 (low)	25	48.2	45.3	6.5
	29	49.2	46.7	4.1
	34	50.1	42.3	7.6
	41	44.0	45.2	10.8
	48	58.5	38.4	3.1
	51	55.1	37.5	7.4
16 (high)	19	51.0	39.4	9.6
	25	46.0	47.5	6.5
	29	44.9	48.1	7.0
	34	46.8	44.6	8.8
	41	45.8	43.5	10.7
	48	57.6	35.3	10.6
	51	65.8	27.1	7.1
17 (high)	15	52.7	39.2	8.1
	25	48.4	45.3	6.3
	29	46.0	48.8	5.2
	34	57.8	33.3	8.9
	41	50.6	41.0	8.4
	48	52.8	41.2	6.0
	51	45.1	43.9	11.0
18 (int)	1	59.3	36.1	4.6
	8	48.9	45.6	5.5
	15	53.3	40.9	5.8
	25	53.7	40.0	6.3

APPENDIX 3B (cont'd).

	29	49.1	44.0	6.9
	34	50.5	44.3	5.2
	41	48.6	45.1	6.3
	48	57.2	37.2	5.6
	51	53.0	39.2	7.8
19	1	50.5	42.2	7.3
	15	46.9	45.4	7.7
	25	44.1	47.2	8.7
	29	51.8	41.2	7.0
	34	52.2	41.0	6.8
	41	51.3	39.5	9.2
	48	50.5	43.5	6.0
	51	52.9	39.5	7.6
20 (low)	1	54.0	40.2	5.8
	8	44.1	49.2	6.7
	15	43.8	45.3	10.9
	25	47.4	46.0	6.6
	29	55.2	42.3	2.5
	34	56.7	39.8	3.5
	48	48.7	43.5	7.8
	51	39.8	50.9	9.3
21 (int)	1	55.3	39.9	4.8
	8	50.5	43.2	6.3
	15	47.0	43.9	9.1
	25	47.7	46.7	5.6
	29	48.4	42.4	9.2
	34	46.4	46.6	7.0
	41	56.7	36.1	7.2
	48	58.0	34.9	7.1
	51	48.4	44.7	6.9
22	8	55.4	38.1	6.5
	15	53.2	39.4	7.4
	25	49.7	44.2	6.1
	29	47.6	47.2	5.2
	34	40.9	47.5	11.6
	41	52.1	39.6	8.3
	48	53.8	39.1	7.1
	51	60.9	34.7	4.4
23 (high)	8	45.8	47.5	6.7
	15	50.5	42.2	7.3
	25	54.5	40.2	5.3
	29	49.2	45.3	5.5
	34	50.6	44.3	5.1
	41	57.6	37.5	4.9
	48	56.6	38.1	5.3
	51	57.1	37.7	5.2
24 (low)	1	54.0	40.5	5.5
	8	51.2	40.2	8.6
	15	47.7	44.3	8.0

APPENDIX 3B (cont'd).

	25	53.0	42.8	4.2
	29	49.2	43.1	7.7
	34	54.8	35.8	9.4
	41	53.9	40.2	5.9
	48	56.3	38.0	5.7
	51	54.2	39.6	6.2
25 (low)	1	51.0	42.4	6.6
	15	48.9	41.3	9.8
	19	51.5	42.3	6.2
	25	49.2	43.9	6.9
	29	53.1	42.4	4.5
	34	51.3	41.7	7.0
	41	55.6	39.7	4.7
	48	58.9	35.8	5.3
	51	51.5	40.3	8.2

CROSS-SECTIONS, 30 µm.

CHIMAERA 115, LEFT AT

FIBRE NO.	SECTION NO.	% GPI-1		
		A	AB	B
1	1	47.3	40.1	12.6
	4	45.3	42.4	12.3
2	1	49.9	39.4	10.7
	5	46.7	40.0	13.3
3	1	47.2	38.0	14.8
	3	44.9	42.1	13.0
	5	47.4	38.4	14.2
4	1	50.6	40.9	8.5
	4	51.9	39.9	8.2
5	1	50.1	40.0	9.9
	3	44.0	42.0	14.0
	4	46.5	42.0	11.5
	5	40.6	45.8	13.6
6	1	49.0	41.0	10.0
	3	46.2	42.1	11.7
	4	45.5	41.1	13.4
	5	42.0	44.2	13.8
7	1	45.3	44.9	9.8
	3	43.9	44.8	11.3
	4	44.4	43.4	12.2
	5	39.7	50.4	9.9
9	1	23.8	51.8	24.4
	4	32.1	43.6	24.3
	5	31.8	47.9	20.3

APPENDIX 3B (cont'd).

11	1	46.3	41.8	11.9
	3	45.3	42.7	12.0
	4	38.8	48.2	13.0
12	1	48.6	38.7	12.7
	4	45.9	39.0	15.1
13	3	42.5	42.9	14.6
	4	39.9	44.8	15.3
	5	47.3	42.1	10.6
14	4	36.8	55.2	8.0
	5	32.4	59.5	8.0

CHIMAERA 115, RIGHT AT

FIBRE NO.	SECTION NO.	% GPI-1		
		A	AB	B
2	1	51.8	42.0	6.2
	2	50.6	40.1	9.3
5	1	48.0	42.7	9.3
	2	49.4	42.9	9.7
9	1	57.3	36.4	6.3
	2	58.2	34.8	7.0
12	1	59.1	33.3	7.6
	2	57.6	32.6	9.8
13	1	58.2	34.7	7.1
	2	59.2	34.6	6.2

CHIMAERA 142, RIGHT AT

FIBRE NO.	SECTION NO.	% GPI-1		
		A	AB	B
3	1	47.9	44.1	8.0
	2	51.6	43.6	4.8
5	1	29.7	54.8	15.5
	2	33.6	48.3	18.1
8	1	58.7	33.9	7.4
	2	56.9	34.3	8.8
9	1	46.0	46.4	7.0
	2	46.0	43.4	10.6
10	1	48.7	46.7	4.6
	2	44.9	46.6	8.5

APPENDIX 3B (cont'd).

CROSS-SECTIONS, 40 µm.

CHIMAERA 115, RIGHT AT

FIBRE NO.	SECTION NO.	% GPI-1		
		A	AB	B
4	1	62.7	32.7	4.6
	2	61.6	34.1	4.3
5	1	70.0	26.9	3.1
	2	64.4	30.8	4.8
6	1	70.3	26.5	3.2
	2	67.7	27.8	4.5

CHIMAERA 123, RIGHT AT

FIBRE NO.	SECTION NO.	% GPI-1		
		A	AB	B
1	1	88.8	10.2	1.0
	2	91.6	7.7	0.7
2	1	89.7	9.2	1.1
	3	90.6	8.9	0.5
3	1	90.2	9.2	0.6
	2	90.1	9.3	0.6
	3	88.9	9.6	1.5
4	1	89.7	9.0	1.3
	2	90.4	9.2	0.4
5	1	89.0	9.9	1.1
	2	91.2	7.6	1.2
	3	89.1	10.1	0.8
6	1	90.4	9.0	0.6
	3	86.6	11.8	1.3
7	1	88.6	11.4	n.d.
	2	88.9	11.1	n.d.
	3	88.2	11.8	n.d.
8	1	85.2	14.8	n.d.
	2	87.5	12.5	n.d.
	3	86.1	13.2	0.7
9	1	82.9	15.3	1.8
	2	86.7	12.0	1.3
	3	84.1	14.4	1.5
10	1	87.7	12.3	n.d.
	2	90.0	10.0	n.d.
	3	85.2	14.2	0.6
11	1	88.1	10.6	n.d.

APPENDIX 3B (cont'd).

	2	90.4	9.6	n.d.
	3	89.4	10.6	n.d.
12	1	89.6	10.4	n.d.
	2	88.7	11.3	n.d.
13	1	93.3	6.7	n.d.
	2	94.4	5.5	0.1
14	1	92.8	7.1	0.1
	2	91.9	7.2	1.9
19	1	92.2	6.8	1.0
	2	89.0	10.6	0.4

CHIMAERA 142, RIGHT AT

FIBRE NO.	SECTION NO.	% GPI-1		
		A	AB	B
4	1	71.4	25.1	3.5
	2	70.3	25.5	4.2
	4	73.5	21.6	4.9
	6	76.3	19.9	3.8
6	1	60.7	29.4	9.9
	2	63.9	28.1	8.0
	4	58.8	33.1	8.1

COLLAGENASE-ISOLATED SEGMENTS

CHIMAERA 115, RIGHT EDL

FIBRE NO.	SEGMENT NO.	% GPI-1		
		A	AB	B
1	1	68.2	29.0	2.8
	2	66.8	29.8	3.4
2	1	76.4	23.5	0.1
	2	76.8	22.8	0.4

APPENDIX 3E (cont'd).

CHIMAERA 116, RIGHT EDL

FIBRE NO.	SEGMENT NO.	% GPI-1		
		A	AB	B
1	1	94.7	5.3	n.d.
	2	94.4	5.6	n.d.

CHIMAERA 117, RIGHT EDL

FIBRE NO.	SEGMENT NO.	% GPI-1		
		A	AB	B
1	1	93.3	6.7	n.d.
	2	95.1	4.9	n.d.
2	1	98.0	2.0	n.d.
	2	98.1	1.9	n.d.

CHIMAERA 190, LEFT AT

FIBRE NO.	SEGMENT NO.	% GPI-1		
		A	AB	B
1	1	54.7	35.7	9.6
	2	58.7	35.4	5.9
	3	64.3	30.3	5.4
2	1	65.3	30.4	4.3
	2	63.4	33.2	3.4
	3	58.6	30.6	10.8
3	1	74.7	19.8	5.5
	2	69.9	26.7	3.4

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