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Evolution of a Gene

Multiple genes for LDH isozymes provide a model of the evolution of gene structure, function, and regulation.

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In the beginning, living systems were relatively simple and depended upon only a small number of macromolecules for metabolic activity and continued existence. Evolutionary progress entailed the acquisition of new macromolecules and metabolic processes, eventually resulting in the great variety and biochemical complexity of the life forms that exist today.

Early biological evolution required the creation of new metabolic machinery encoded in new deoxyribonucleic acid (DNA). However, much subsequent evolutionary change has depended primarily upon the modification and elaboration of preexisting components, particularly the DNA (*1, 2*), as indeed the term evolution implies. Thus, large portions of the genetic information (the DNA) are internally homologous within single organisms and also between organisms, even between those that are only distantly related (*3*). Correspondingly, protein molecules engaged in similar tasks, either in the same or in different organisms, are likely to be truly homologous.

Unfortunately, the historical process of biochemical diversification based on the evolution of genes cannot be examined directly since the biochemical record of the past has been largely obliterated. However, the probable course of gene evolution can be reconstructed by examining a wide spectrum of related organisms with reference to the synthesis and activity of specific proteins. For example, the extensive analysis of the primary structure (amino acid sequence) of the cytochrome c molecules of a large number of animals, plants, and protists has revealed that all of these proteins are, indeed, very similar to one another (*4, 5*). These data have allowed the construction of a phylogenetic tree for cytochrome c and a simulation of the evolutionary history of the gene locus encoding this protein (*6*). It is possible, by using these procedures of reconstruction, to document indirectly the number, variety, and timing of mutational events experienced by this gene during its evolution and thereby to explain the array of cytochrome c molecules characteristic of living organisms today.

During the course of evolution, new metabolic functions and new protein molecules have come into existence. Since a sequence of about 1000 nucleotides is required to encode an average-sized protein, the probability of a functional protein arising anew seems infinitely small. In contemporary organisms it is highly unlikely that the random generalization of a new sequence of nucleotides would result in the transcription of a functional messenger ribonucleic acid (mRNA), let alone lead to the production of a protein having any metabolic significance. Novel nucleotide sequences must have been generated and tested billions of years ago during the earliest evolution of biological systems, but the creation of totally new sequences of nucleotides can scarcely be a significant mechanism for generating new information in highly complex and integrated organisms such as those existing today. Far more likely now is the derivation of new genetic information by duplication and subsequent modification of previously existing information, that is, from functioning genes (*7, 8*). Such a duplication of genes coding for specific proteins could be followed by mutational changes eventually resulting in proteins of somewhat different structure and, therefore, different function. A duplicated gene coding for a specific enzyme, for example, could gradually be changed

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by mutational events to produce an enzyme with a broadened substrate specificity and then, finally, the specificity could be narrowed again to focus on an entirely new substrate (*9*). Such a process of evolutionary transformation of one enzyme into another would fulfill the evolutionary requirement that intermediates have selective value in order to persist.

Intensive research in many laboratories has led to the identification of amino acid sequences and has generated other biochemical data that have made possible the recognition of structural homologies among many different proteins within a species as well as between species (*5, 10*). Extensive analyses of amino acid sequences of vertebrate globins (myoglobin and hemoglobin) have documented the homologous nature of these proteins and demonstrated that the gene for myoglobin and those for hemoglobin (α, β, γ, and δ) are all related (*11*). Similarly, the immunoglobins (*12*), histones (*13*), many serine proteases (*14*), α-lactalbumin and lysozyme (*15*), the ferredoxins (*5*), and several dehydrogenases (*16, 17*) all exhibit extensive sequence homology. Recently, the study of protein conformation, particularly the three-dimensional structure of binding domains, has proved to be an especially powerful method for detecting distant evolutionary homologies among proteins (*18*). These various data support the hypothesis that most proteins have arisen by gene duplication from a small number of "ancestral genes" (*7, 19, 20*).

Great steps in evolution require the acquisition of new genetic information, but evolutionary divergence and specialization, as among closely related taxa, is apparently achieved by changes in the use or expression of essentially the same genetic information. Since the basic biochemistry of life is similar in all organisms, metabolic reactions, particularly in related groups such as the vertebrates, must generally be dependent upon the same kind of genetic information, as expressed in specific enzymes and other proteins. It is true that homologous proteins may vary somewhat in amino acid composition from species to species, but their basic biochemical properties and metabolic roles remain fundamentally the same. What distinguishes one vertebrate from another are primarily changes in the timing of expression and the relative amounts of the same gene products, not the minor differences evident in the structure of these gene products. This seems obvious in closely related individuals. The biochemical and morphological differences among members of the same species, for example, are based largely on the timing and amount of gene activity, rather than upon qualitative differences in the structural genes themselves or their protein products. Some qualitative differences have, of course, been described, but they do not appear to be essential in distinguishing one individual from another. Thus, a substantial amount of evolutionary change, perhaps most of the diversification in related taxa, may be based upon changes in the regulation of the function of structural genes rather than upon changes in those genes themselves.

Both the quantity and quality of protein synthesis and enzymatic activity change extensively in an orderly, programmed sequence during the course of ontogeny (*21–23*). Thus, the differentiated cells and tissues of adult organisms normally contain characteristically different repertories of proteins in accord with their metabolic activities (*24–27*). Since these quantitative differences in enzyme content are inherited, they must be based on the genome, perhaps in the reiterated DNA (*28*). Interference with the normal program of differential gene expression, either by specific inhibitors (*29*) or by genomic malfunction (cancer or mutations, for example) (*30*), results in impaired function and development, and perhaps death of the organism. These observations suggest that the forces of natural selection act not only on the structure and function of a gene and its protein product, but also on the regulation of gene function. Thus, the evolution of a gene involves two distinct processes. First, a new gene arises by duplication from an original gene and then it diverges from that gene by the accumulation of mutations which alter its structure and correspondingly the structure and function of its product. The second part of the evolutionary process involves changes in regulation (*31*) so that this new gene is expressed at those times and in those cells for which it is advantageous and is not expressed when it would be detrimental.

Previous investigations into molecular evolution have dealt almost exclusively with changes in structure and function. In these studies proteins such as cytochrome c, hemoglobins, fibrinopeptides, and immunoglobins have received the most attention. However, except for the hemoglobins, these particular proteins have not proved very useful for investigating changes in gene regulation. The complete process of gene evolution (changes in structure, function, *and* regulation) can probably best be examined through the study of multilocus isozyme systems (*32*). Isozymes are multiple molecular forms of an enzyme in a species (*24*). The enzyme lactate dehydrogenase (LDH; E.C. 1.1.1.27) exists in several such isozymic forms and provides an informative system for examining gene evolution.

Lactate Dehydrogenase Structure

The enzyme LDH presides over the interconversion of pyruvate and lactate in the glycolytic pathway and thereby serves as an important source of the oxidized coenzyme [nicotinamide adenine dinucleotide (NAD)] during periods of transient anaerobiosis. Invertebrates, protists, and bacteria have a variety of lactate dehydrogenases, some specific for D-lactate and some for L-lactate; these LDH's range in molecular weight from 70,000 to about 140,000 and exhibit a variety of unusual kinetic properties (*33*). The evolutionary relationships among the many LDH's of lower organisms and, in turn, their possible relationship to the lactate dehydrogenases of vertebrates are at present unknown, but extensive homology seems unlikely. Although it would be interesting to know the interrelationships of invertebrate and vertebrate LDH's, the data necessary for such comparisons have not yet been obtained.

In contrast to the heterogeneous and poorly understood assemblage of enzymes catalyzing the pyruvate-lactate interconversion in lower organisms, the lactate dehydrogenase isozymes of vertebrates comprise a single homologous family. Like many enzymes, LDH exists in a variety of isozymic forms, all related in that they catalyze the same chemical reaction, but all different from one another in molecular structure and commonly in genetic control. We believe that only a single gene encoded the LDH polypeptide at the beginning of vertebrate evolution. The subunits encoded by this gene were able to polymerize to make a homotetramer with properties similar to those of the A_4 isozyme commonly found now in the skeletal muscle of all vertebrates. Since all contemporary vertebrates examined, save one, have at least two genes coding for LDH polypeptides, the original A gene must have soon duplicated. Later these two A-like genes diverged by mutation to give rise to two distinctly different genes now designated A and B (*34*). The corresponding polypeptide subunits encoded by these two structural genes commonly associate at random to generate binomial distributions of the expected five tetrameric isozymes having the following subunit compositions: A_4, A_3B_1, A_2B_2, A_1B_3, and B_4 (*22, 25, 35, 36*). These different isozymic forms are readily separated by electrophoresis and can be subsequently visualized as five distinct bands by a specific LDH staining reaction. This two-gene, five-isozyme system characterizes mammals and birds. All other vertebrates likewise possess these two genes for LDH but unrestricted polymerization of their protein products does not always occur.

Table 1. Characteristics of fish lactate dehydrogenases. Relative quantities of C subunits: +++, most abundant; ++ and +, intermediate abundance; ± marginal presence; –, undetectable; blank, tissue not examined. Abbreviations: RAM, relative anodal mobility; Mu, muscle; He, heart; Br, brain; St, stomach; Gi, gills; Li, liver; Sp, spleen; Go, gonad; Ki, kidney

Fish species	A–B tetramers (No.)	C subunit	RAM	Expression of the LDH C locus									
				Mu	He	Eye	Br	St	Gi	Li	Sp	Go	Ki
				Class Agnatha									
Order Myxiniformes													
Atlantic hagfish (*Myxine glutinosa*)	2 or more	No	B>A	–	–	–	–	–		–	–	–	–
Order Petromyzontiformes													
Sea lamprey (*Petromyzon marinus*)	1	No	A	–	–	–	–	–	–	–	–	–	–
American brook lamprey (*Lampetra lamottei*)	1	No	A	–	–	–	–	–	–	–	–	–	–
				Class Chondrichthyes									
Order Squaliformes													
European dogfish (*Mustelus mustelus*)	4	No	B>A	–	–	–	–	–	–	–	–	–	–
Reef shark (*Carcharhinus springeri*)	4	No	B>A	–	–	–	–	–	–	–	–	–	–
Spiny dogfish (*Squalus acanthias*)	5	No	B>A	–	–	–	–	–	–	–	–	–	–
Order Rajiformes													
Clearnose skate (*Raja eglanteria*)	2–5	No	B>A	–	–	–	–	–	–	–	–	–	–
Bullnose ray (*Myliobatis freminvillei*)	4–5	No	B>A	–	–	–	–	–	–	–	–	–	–
Order Chimaeriformes													
Ratfish (*Hydrolagus colliei*)	2	No	B>A	–	–	–	–	–	–	–	–	–	–
Order Dipnoi				*Class Osteichthyes*									
South American lungfish (*Lepidosiren paradoxa*)	2–3	No?	B>A	–	–	–	–	–	–	–	–	–	–
Order Acipenseriformes													
Pallid sturgeon (*Scaphirhynchus albus*)	5	Yes	B>A>C	–	+	–	+	+	++	+++	+	+	+
Paddlefish (*Polyodon spathula*)	5	Yes	B>C>A	–	–	–	±	–	+	+	++	–	+++
Order Amiiformes													
Bowfin (*Amia calva*)	2–3	Yes	C>B>A	+	+	++	+++	+	+	+	+	+	+
Order Elopiformes													
Bonefish (*Albula vulpes*)	4–5	Yes	C>B>A	–	–	–	–	–	+	+	++	–	+++
Order Anguilliformes													
American eel (*Anguilla rostrata*)	2–3	Yes	B>A>C	–	–	+	–	+	+	±	+	±	+++
Spotted moray (*Gymnothorax moringa*)	3	Yes	C>B>A	+	++	+	+	+	++	+	+++	+	++
Order Osteoglossiformes													
Butterfly fish (*Pantodon buchholzi*)	2	Yes	A>B>C	–	++	+	–	++	+	+	+	+	+++
Green aruana (*Osteoglossum bicirrhosum*)	5	Yes	C>B>A	–	–	++	+++	–	+	–	–		–
African knifefish (*Xenomystus nigri*)	2	Yes	B>A>C	–	–	–	–	–	–	+++	–	–	+
Order Clupeiformes													
Blueback herring (*Alosa aestivalis*)	5	Yes	B>C>A	–	–	+++	++	–	–	–	–	–	–
Atlantic menhaden (*Brevoortia tyrannus*)	5	Yes	B>C>A	–	–	+++	++	–	–	–	–	–	–
Atlantic herring (*Clupea harengus harengus*)	3	Yes	B>C>A	–	–	+++	–	–	–	–	–	–	–
Round herring (*Etrumeus teres*)	3	Yes	B>C>A	–	–	+++	++	–	–	–	–	–	–
Order Salmoniformes													
Rainbow trout (*Salmo gairdneri*)	2	Yes	C>B>A	–	–	+++	++	+	–	+	–	–	–
Brook trout (*Salvelinus fontinalis*)	2	Yes	C>B>A	–	–	+++	+	–	–	–	–	–	–
Rainbow smelt (*Osmerus mordax*)	2	Yes	B>A>C	–	+	+++	+	–	+	–			

Fish species	A–B tetramers (No.)	C subunit	RAM	Expression of the LDH C locus									
				Mu	He	Eye	Br	St	Gi	Li	Sp	Go	Ki
Grass pickerel (*Esox americanus vermiculatus*)	3	Yes	C>B>A	+	++	+++	++	+	+	+	±	++	+
Order Myctophiformes													
Sand diver (*Synodus intermedius*)	2–3	Yes	C>A>B	–	–	+++	++	±	–	–			±
Order Cypriniformes													
Goldfish (*Carassius auratus*)	2–5	Yes	B>A>C	–	+	+	++	±	±	+++	±	++	+
Carp (*Cyprinus carpio*)	2–5	Yes	B>A>C	–	–	–	–	–	–	+++	–	–	–
Striped shiner (*Notropis chrysocephalus*)	5	Yes	B>A>C	–	–	–	–	–	–	+++	+	++	±
Bluntnose minnow (*Pimephales notatus*)	5	Yes	B>A>C	–	+	+	±	±	–	+++	+	++	+
Quillback (*Carpiodes cyprinus*)	5	Yes	B>A>C	–	–	–	–	–	–	+++	+	+	–
White sucker (*Catostomus commersoni*)	5	Yes	B>A>C	–	–	–	±	–	–	+++	–	–	–
Northern hog sucker (*Hypentelium nigricans*)	5	Yes	B>A>C	–	±	–	+	+	–	+++	+	±	+
Order Percopsiformes													
Pirate perch (*Aphredoderus sayanus*)	3	Yes	C>A>B	–	–	+++	+	–	–	–	–	–	–
Order Gadiformes													
Atlantic cod (*Gadus morhua*)	2–3	Yes	B>A>C	–	+	++	±	+	±	+++	+	+	+
Cusk (*Brosme brosme*)	2	Yes	A>B>C	–	+	+	–	++	–	+++	+	+	+
Silver hake (*Merluccius bilinearis*)	2–3	Yes	B>A>C	–	+	++	+	++	+	+++	++	++	+
Order Atheriniformes													
Ballyhoo (*Hemiramphus brasiliensis*)	3	Yes	C>A>B	–	–	+++	++	–	–	–	–	–	–
Houndfish (*Tylosaurus crocodilus*)	3	Yes	C>B>A	–	–	+++	++	–	–	–	–	–	–
Order Beryciformes													
Longspine squirrelfish (*Holocentrus rufus*)	3	Yes	C>B>A	±	+	+++	++	+	±	+	±	++	+
Order Perciformes													
Nassau grouper (*Epinephelus striatus*)	5	Yes	C>A>B	–	–	+++	++	–	–	–	–	–	–
Bluefish (*Pomatomus saltatrix*)	2	Yes	C>B>A	–	–	+++	++	–	–	–	–	–	–
Sharksucker (*Echeneis naucrates*)	3	Yes	C>A>B	–	–	+++	++	+	–	–	–	–	–
Spotfin mojarra (*Eucinostomus argenteus*)	3	Yes	C>B>A	–	–	+++	++	–	–	–	–	–	–
Spotted goatfish (*Pseudopeneus maculatus*)	2	Yes	C>B>A	–	–	+++	++	–	–	–	–	–	–
Rainbow parrotfish (*Scarus guacamaia*)	3	Yes	C>B>A	–	+	+++	+	–	–	–	–	–	–
Great barracuda (*Sphyraena barracuda*)	3	Yes	C>B>A	–	±	+++	++	–	–	–	±	–	±
Hairy blenny (*Labrisomus nuchipinnis*)	3	Yes	C>A>B	–	–	+++	++	–	–	+		–	–
Frillfin goby (*Bathygobius soporator*)	3	Yes	C>B>A	–	–	+++	++	–	–	–	–	–	–
Blue tang (*Acanthurus coeruleus*)	3	Yes	C>A>B	–	–	+++	++	–	–	±	–	–	–
Order Pleuronectiformes													
Summer flounder (*Paralichthys dentatus*)	2	Yes	C>A>B	–	–	+++	++	–	–	–			
Winter flounder (*Pseudopleuronectes americanus*)	3	Yes	C>B>A	–	–	+++	++	–	±	–	–	–	–
Order Tetraodontiformes													
Gray triggerfish (*Balistes capriscus*)	3	Yes	C>B>A	–	–	+++	++	+	+	+	–	–	–
Scrawled cowfish (*Lactophrys quadricornis*)	5	Yes	C>B>A	–	–	+++	++	–	–	–	–	–	–
Balloonfish (*Diodon holocanthus*)	3	Yes	C>A>B	–	–	+++	++	–	–	–	–	–	–

Evolution of Structure of A and B Subunits

Although originally identical, the present A and B subunits of LDH are now quite distinct proteins, though retaining extensive homology. One line of evidence for this homology is the ability of these subunits to interact to form enzymatically active tetramers. This association occurs in vivo, as mentioned above, and can be induced in vitro with the LDH subunits of almost any two vertebrate species, even from different vertebrate classes (*36, 37*). These data indicate that despite the extensive divergence of the A and B subunits in many characteristics, the intersubunit binding sites have been strongly conserved throughout evolution. Homology of the LDH A and B subunits is also suggested by the virtually identical amino acid sequences of the "active-site peptides" from a variety of vertebrates (*17, 38*). Furthermore, immunological investigations have demonstrated homology between these two subunits (*39–41*).

Superimposed upon this ancient homology of the LDH A and B subunits is the extensive divergence in structure and function of these subunits that has accompanied the evolutionary progression of vertebrate species. The purified A_4 and B_4 homopolymers and, thus, the A and B subunits, differ in many characteristics. As already mentioned, the isozymes of LDH are readily resolved by electrophoresis and their differing mobilities reflect differences in net charge and, therefore, differences in amino acid composition. The amino acid composition and peptide maps of the LDH's from a large number of vertebrates reveal extensive variation in the primary structure of these subunits (*42–45*). The general lack of immunological cross-reactivity of the A and B subunits dramatically emphasizes this structural divergence (*46, 47*). Furthermore, detailed comparisons have demonstrated that such enzymatic characteristics as the Michaelis constant K_m, substrate concentration optima, reactivity with coenzyme analogs, and binding and elution properties of these two kinds of subunits are strikingly different (*43, 44, 48–50*). In fact, these measurements demonstrate that the A subunits from all vertebrates are related to one another as a group and are quite distinct from the B subunits, which also are clearly related. These observations indicate that the A and B subunits are only distantly homologous and also that the subunits diverged rather early to occupy separate metabolic niches. Subsequent selection has maintained the differences in structure of each of these two subunits to maximize the efficiency of each in their respective roles. Thus, each of these LDH subunits is highly tailored for its specific function or functions. Such specialization, although maximizing metabolic efficiency, carries a price in evolutionary rigidity.

Because all vertebrate LDH's are tetramers, even those of the most primitive vertebrates existing today, the primordial A and B subunits would have associated with each other to generate tetrameric enzyme molecules. As the nucleotide sequences of the two LDH genes diverged through time by the slow accumulation of mutations, the corresponding subunits became dissimilar but would still be expected to polymerize at random to generate all possible tetrameric combinations. Allelic variants of LDH subunits have been reported in many species (*26, 51*), and these nearly identical LDH subunits, which freely associate as tetramers, serve as a model for the behavior of the ancestral subunits at the time of their origin. Indeed, as mentioned above, observation and analysis of the interaction of the A and B subunits of mammals and birds (although these subunits differ extensively in physical, kinetic, and immunochemical properties) reveals that even these quite distinct subunits nevertheless associate readily to generate all of the five possible tetrameric isozymes.

Although many species of fishes do exhibit unrestricted association of A and B subunits, the majority do not (Table 1) (*26, 50, 52, 53*). Thus, restricted LDH subunit association is common in fishes and similar, though less extensive, data suggest the same situation in some amphibians and reptiles. These observations may be explained by assuming that the intersubunit binding sites of these subunits have varied during evolution so that specific subunit associations within certain species are forbidden while free association with subunits of other distantly related species is still possible. This is strikingly different from the situation observed in mammals and birds and suggests a fundamental difference between the LDH's of poikilotherms and homeotherms. In the latter group, natural selection has maintained an enzyme structure that permits random subunit association, while in the lower vertebrates random subunit association has not always been maintained and has, apparently, been expressly selected against in some species.

It seems reasonable to assume that as long as the products of two different genes must interact to form functional molecules (for example, the LDH heteropolymers) the evolution of each gene is tied to that of the other. Thus, such coupled genes must, to some extent, coevolve since changes in one will affect the function of the product of the other. However, prevention of subunit association changes this relationship and allows natural selection to act on each gene more or less independently. Such a situation presumably also occurs during the divergence of two homologous forms of the same enzyme to yield two separate enzymes acting upon different substrates.

The LDH's of fishes differ from those of other groups of vertebrates in another fundamental property—net charge. Both the absolute and the relative net charge on the A and B subunits of LDH tend to be somewhat different in each class of vertebrates. In all mammals, nearly all birds, and most reptiles and amphibians (*54*), electrophoretic analysis reveals that the B subunit is considerably more negatively charged than the A subunit. Moreover, the B subunits of many groups (especially mammals) have very similar net charges, and the same is true of the A subunits. Fishes, on the other hand, exhibit a great deal more variation in both the absolute and the relative net charges on the A and B subunits (*26, 44, 50, 52*). As summarized in Table 1, this variation is so extreme that the relative net charges on the A and B subunits are reversed in many species of fishes so that the A subunit is more negatively charged than the B subunit.

Evolution of Regulation of A and B Genes

As a general rule, vertebrates contain high levels of both A and B subunits in most tissues. Thus, both genes are active. Each tissue, however, has its own characteristic pattern of isozymes, in terms of relative abundance, and this abundance reflects the balance of synthesis and degradation for each kind of subunit (*21, 26, 55*). The general expression of the LDH A and B genes is remarkably uniform throughout the vertebrates. Thus, A subunits predominate in white skeletal muscle while the B subunits predominate in tissues such as heart muscle and brain. This uniformity in the specificity of gene expression strongly suggests the suitability of the gene products for the specific metabolic roles characteristic of each tissue. Moreover, the pattern of LDH gene activity during early development appears quite similar in many vertebrates. In almost all mammals, birds, amphibians, and fishes investigated, the B subunits predominate in the unfertilized egg and through early cleavage; then an abrupt increase in A subunits occurs, followed in later stages of development by a resurgence of synthesis of B subunits (*23, 56*).

In contrast to this general picture, some

groups of fishes display a different pattern of function of the A and B genes. Among the advanced teleosts, certain families in the order Perciformes and all families that have been examined in the orders Pleuronectiformes and Tetraodontiformes exhibit a sharp reduction in the relative activity of the B gene; nearly all tissues contain only the A_4 isozyme. For a long time it was believed that these were truly "one-isozyme" fishes containing only the A gene for LDH, the B gene having been lost (*25, 52, 57*). Recently, however, detailed examinations have clearly demonstrated both A and B subunits in these fishes (Table 1) (*44, 58*), but the B gene is active in only a few tissues such as eye and brain and even then contributes only a minor part of the total LDH activity. These advanced orders of teleosts have abandoned much of the LDH isozyme variety genetically available to them. This simplification of isozyme patterns is surprising and perplexing. These fishes are relatively inactive compared to many other teleosts, but neither the patterns of activity nor the nature of their physiology provide any obvious explanation for the reduction in the activity of the B gene. A greater understanding of the functional significance of the different isozymes will be required before these changes in tissue expression can be explained.

Various species of mammals provide additional insight into evolutionary changes in LDH gene regulation. For example, throughout the course of primate evolution there appears to have been a general increase in the relative expression of the B gene over the A gene in nearly all tissues (*59*). In contrast, certain rodents are characterized by a restriction of expression of the LDH B gene in erythrocytes (*60*). Various inbred mouse strains exhibit low levels of LDH B subunits in red blood cells while several other strains completely lack B subunits in these cells. The patterns of LDH A and B subunit synthesis in other tissues of these mice are unchanged. This trait, reduction of B subunits in erythrocytes, is inherited as an autosomal dominant. Evolutionary surveys have further shown that certain families of rodents have characteristically different levels of B subunits in their erythrocytes. The LDH patterns of other tissues of these rodents are virtually identical. These data suggest that there is a regulator gene which specifically controls the expression of the B gene in rodent erythrocytes. Clearly, the LDH A and B genes of rodents are subjected to differential and highly specific regulation. Such regulatory control is undoubtedly subject to mutational change and natural selection just as is true for structural genes.

The C Subunit of Mammals and Birds

Both the LDH A and LDH B genes and their protein products have been tailored by selective pressures during evolution to function efficiently in specific metabolic roles. Additional major evolutionary changes in these two genes would have been strongly resisted as soon as their products fulfilled essential roles in metabolism. One obvious way to circumvent such restraints on further enzyme evolution would be to duplicate one of the genes; the new product would then be available for evolutionary modification without affecting the previous role of the parental gene and its enzymic product. Such a second duplication event among the LDH genes has indeed occurred at least once in the vertebrates and given rise to a third LDH gene which richly traces the course of gene evolution. In mammals and birds this gene, LDH C, encodes subunits which readily hybridize with both A and B subunits (*61*); all three genes are therefore homologous.

Structurally and functionally the C subunit, like the A and B subunits, has its own characteristic properties. The C_4 isozyme (or LDH-X as it is sometimes called) has an amino acid composition and yields peptide maps similar to, but distinct from, those of the other LDH's (*43, 62*). Kinetic properties of this isozyme are likewise distinct from those of the A_4 and B_4 isozymes, especially with regard to substrate utilization. Like the A_4 and B_4 isozymes, the C_4 is indeed a lactate dehydrogenase and acts primarily on lactate and pyruvate as substrates. Many of the C_4 isozymes exhibit a much broader substrate specificity than do other LDH's. These isozymes are able to catalyze efficiently the transformation of a number of related compounds, namely, other α-hydroxy acids such as α-hydroxybutyrate or α-hydroxyvalerate, compounds which do not serve as substrates for the A or B isozymes (*63*).

Several lines of evidence suggest at least indirectly that the C gene of homeotherms was derived by duplication from the B gene. The physical, kinetic, and immunochemical properties of the C subunit generally resemble more closely those of the B subunit than those of the A subunit (*41, 49, 64, 65*). Furthermore, the report that the LDH B and C loci of pigeons are closely linked, perhaps even contiguous (*66*), suggests that the C gene arose by a tandem duplication of the B gene.

The observation that the B and C loci are very closely linked is important when considering the regulation of these two genes. The B gene of homeotherms is characteristically expressed in nearly all tissues. Unlike the A and B genes of LDH, the C gene of mammals and birds exhibits extreme restriction in both temporal and cellular expression, being active only in the primary spermatocytes of sexually mature males (*63, 65, 66*). The apparent close linkage of the B and C genes does not prevent the independent and remarkably dissimilar regulation of these two genes. Does the differential expression of these two genes stem from associated controlling elements which may be radically different, or does it result from the simple divergence in the nucleotide sequences of the structural genes themselves?

Contrary to previous notions, the C_4 isozyme of mammals and birds is not the only LDH which exhibits a broad substrate specificity. As shown in Fig. 1, the B_4 isozyme of certain reptiles is also characterized by high reactivity with other α-hydroxy acids. This result was obtained for the B_4 isozymes of a number of species of reptiles including lizards and alligators. These data indicate that the C gene of birds and mammals probably evolved directly from a duplicated ancestral B gene. Moreover, these data also suggest that the C gene is not unique to mammals and birds, but may exist in somewhat different form in lower vertebrates.

The C Subunit of Fishes

Soon after the discovery of the LDH C gene of mammals and birds it was shown that some teleost fishes also possess a third LDH gene (*52, 67*). Several studies have revealed that this gene is widespread among teleosts and that, like the C_4 isozyme of mammals and birds, its product exhibits a number of specialized properties. Most notably, in many species this isozyme is characterized by a large net negative charge at *p*H 7 and by restriction to neural tissues, namely eye and brain. On electrophoretic resolution of eye homogenates of most fishes this isozyme migrates rapidly toward the anode. The subunits of this "eye-band" of LDH are synthesized primarily in the ellipsoid region of the photoreceptor cells and first appear at the time of retinal differentiation (*50, 68, 69*). Evolutionary surveys of fishes have shown that not all species possess an anodal "retinal-specific" eye-band of LDH (*40, 70, 71*). Some possess an eye-band with different electrophoretic properties. It may lie within the area of migration of the A and B isozymes or even be cathodal in location. Moreover, some groups lack this eye-band entirely but do possess a third LDH gene expressed in the liver though not in the eye (*69, 72–74*). These observations and the extensive survey reported below indicate that

virtually all bony fishes possess three LDH genes. Our investigations employing immunochemical, genetic, physical, and phylogenetic approaches have demonstrated that the eye-band LDH seen in many groups of teleosts and the liver-band LDH seen in other groups are encoded in the same basic locus, even though the isozymic products have somewhat different properties in these different groups. The eye-band LDH has variously been called E_4, C_4, or retinal isozyme 1, while the liver-band has been referred to as C_4, D_4, F_4, L_4, or liver-specific LDH. We propose that because of their common genetic basis all these isozymes should be referred to as the C_4 isozyme. Thus, in parallel to the nomenclature for birds and mammals, the LDH genes of fishes should be designated A, B, and C. Confirmation of the homology of the C genes of vertebrates must await further studies of the enzymes of homeotherms and poikilotherms, particularly amphibians and reptiles.

The teleost C subunit is clearly related to the A and B subunits of LDH; all of these subunits readily associate to generate active tetrameric enzyme molecules. In addition, like the C subunit of mammals and birds, the C subunit of teleost fishes is much more like the B than the A subunit with regard to kinetic, physical, and immunochemical properties (*39, 50, 69, 73–77*). These properties indicate that the C gene of fishes arose from the B gene by a single duplication event (*39, 50, 76, 77*; but see also *74*). Because of the highly diverse and rich patterns of LDH isozymes exhibited by fishes, a detailed analysis of the evolution of fish LDH isozymes was undertaken and the results are presented below.

The Evolution of Fish LDH's

We have examined the LDH's of representatives of the three most primitive classes of vertebrates, the Agnatha (hagfishes and lampreys), the Chondrichthyes (sharks, rays, skates, and ratfishes), and the Osteichthyes (bony fishes). The genetic basis for the LDH isozymes of these lower vertebrates is certainly analogous to and in most cases clearly homologous to that of the higher vertebrates. The genetic constitution (with regard to LDH genes) of the most primitive vertebrates—agnathans and also cartilaginous fishes—seems to be ancestral to that of the more advanced bony fishes and the higher vertebrates.

In the class Agnatha, representatives of both the order Petromyzontiformes (lampreys) and the order Myxiniformes (hagfishes) were investigated. Analysis of the isozyme patterns in these primitive vertebrates reveals the presence of two genes encoding LDH subunits in the hagfish while only one gene for LDH could be detected in the lamprey (Fig. 2a). The single LDH of the lamprey, like all other vertebrate LDH's, is a tetramer that can be dissociated in vitro and recombined with subunits from other species of vertebrates. This isozyme, as tested by immunochemical reactivity, appears homologous to the A subunit of other fishes. Thus, lampreys may resemble the most primitive ancestral vertebrate in having only one gene for LDH. Alternatively, an earlier two-gene condition may have been transformed into the lamprey pattern by loss of the B gene. In any event, it is clear that one isozyme of LDH is sufficient to fulfill the metabolic requirements of all the different

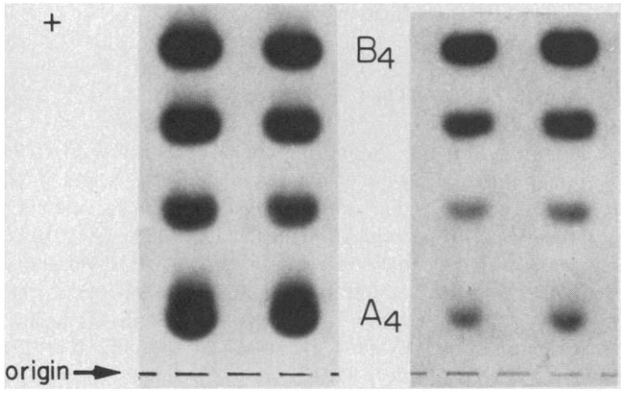
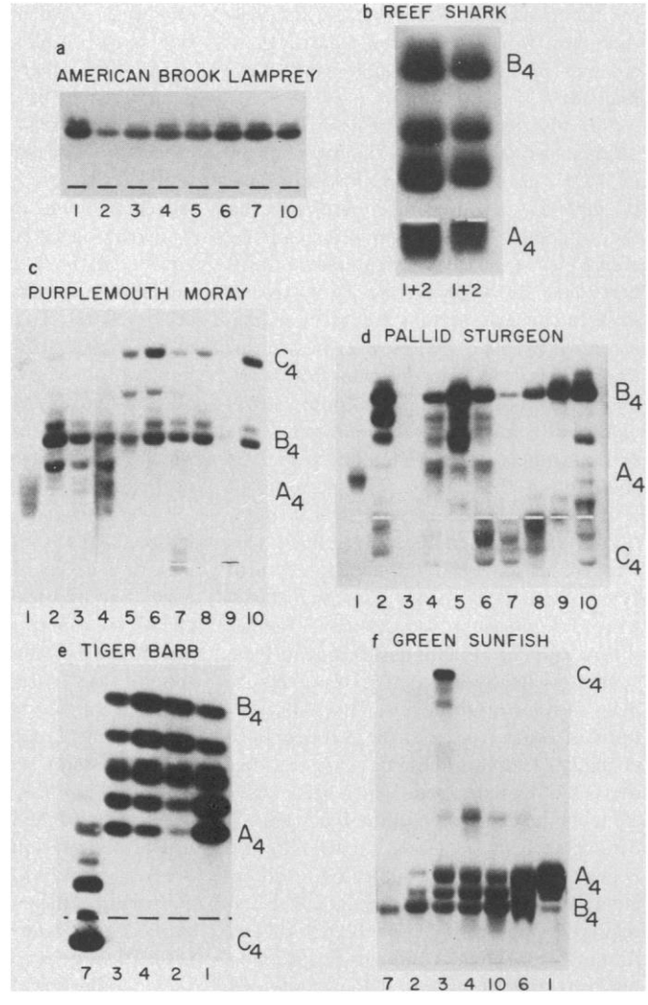


Fig. 1 (above). Substrate specificity of lizard (tokay gecko) LDH isozymes. The isozymes were separated by subjecting an extract of skeletal muscle and heart muscle to vertical starch gel electrophoresis (12 percent Electrostarch; EBT buffer [see (*94*)], *p*H 8.6; 225 volts for 20 hours). Following electrophoresis, the LDH isozymes were visualized by specific histochemical staining (0.25 m*M* NAD, 0.24 m*M* PMS, 0.10 m*M* NBT, and 50 m*M* substrate which was either L-lactate (left) or α-hydroxybutyrate (right). Fig. 2 (right). Lactate dehydrogenase isozyme patterns of fishes. (a) American brook lamprey (*Lampetra lamottei*) possessing only one major isozyme, A_4; (b) reef shark (*Carcharhinus springeri*) LDH composed of four isozymes made up of two different subunits, A and B; (c) purplemouth moray (*Gymnothorax vicinus*) isozymes containing LDH A, B, and C subunits (note presence of C subunits in many tissues); (d) pallid sturgeon (*Scaphirhynchus albus*) isozymes containing A, B, and C subunits (note presence of C subunits in many tissues); (e) tiger barb (*Barbus sumatranus*) isozymes, note cathodal C_4 in liver; (f) green sunfish (*Lepomis cyanellus*) isozymes, note very anodal C_4 in the eye. 1, White skeletal muscle; 2, heart; 3, eye; 4, brain; 5, stomach; 6, gills; 7, liver; 8, spleen; 9, gonad; 10, kidney.



kinds of cells found in a lamprey. Although the hagfish in certain characteristics is apparently even more primitive than the lamprey, two genes are required to generate the LDH isozyme patterns characteristic of this species. No clear evidence has been obtained, however, for the existence of a third gene. These results are in accord with the proposed scheme of evolution of LDH genes and provide examples of two major steps in that evolution, namely the existence of primitive vertebrates having at first one and then two LDH genes.

The next group in the scale of evolution is the cartilagenous fishes of the class Chondrichthyes, of which sharks (in the order Squaliformes) are representative examples. Sharks have been analyzed in detail, and their isozyme patterns demonstrate that at least two genes are involved. Many sharks, such as the reef shark (Fig. 2b), exhibit only four isozymes rather than the expected five after electrophoretic resolution of tissue homogenates. The dogfish shark, however, exhibits the normal five-isozyme pattern. Dissociation and recombination of the two isozymes at the extreme ends of the spectrum (homopolymers A_4 and B_4) as obtained from the reef shark generate two intermediate forms and only two. We must conclude on the basis of this evidence that this shark has two genes for LDH, but that the combination of their corresponding polypeptide subunits is unusual in that only two heteropolymeric isozymes are formed instead of the expected three. Such "four-isozyme" patterns have also been observed in some bony fishes and some lizards, so they are not unique to the sharks.

Because the existing cartilagenous fishes are so distantly related to present-day bony fishes, it is difficult to demonstrate precise homologies between the LDH isozymes of these two groups of fishes. Antiserums against either A or B subunits of an advanced teleost interact approximately equally well with all of the isozymes of the shark. Nevertheless, on the basis of tissue specificity and differential behavior on affinity chromatographic columns one can confidently assign the subunit composition A_4 to the most cathodal isozyme (which predominates in white skeletal muscle) and B_4 to the most anodal (which predominates in heart muscle). The composition of the two intermediate isozymes remains uncertain, but each must be a heteropolymer containing both kinds of subunits. Another member of this class, the ratfish (in the order Chimaeriformes), also possesses two homopolymeric isozymes of LDH corresponding to the A_4 and B_4 isozymes of higher vertebrates. Antiserums against the A_4 isozyme of higher teleosts do, in fact, react somewhat preferentially with one of the isozymes of the ratfish and, therefore, allow us to assign the A_4 composition to this isozyme. These immunochemical homologies also correspond to identifications based on tissue distribution, the A_4 always being predominant in white skeletal muscle and usually relatively cathodal in electrophoretic mobility.

The bony fishes (class Osteichthyes) are evolutionarily more advanced than the two classes of fishes already discussed and, in fact, represent the line from which the higher vertebrates (amphibians, reptiles, birds, and mammals) descended. Because the bony fishes occupy this rather central position with regard to vertebrate evolution, the pattern and variety of LDH isozymes in this group is of critical importance to the present discussion. The LDH's of these fishes tell us much about the course of gene evolution.

Among the bony fishes there have been two major lines of evolution, one leading directly to the advanced fishes including the teleosts and the other leading to the higher vertebrates (amphibians, for example). This second branch of the bony fishes leading to the land vertebrates is poorly represented by existing forms; only the lungfishes and the coelacanth are living today. Although we have investigated the LDH isozyme patterns of the lungfish and, in fact, the coelacanth also, it is not possible to provide a complete interpretation of our results because of both technical problems and a shortage of material. We can, however, conclude that each does have at least two genes for LDH. No satisfactory evidence for a third gene in these fishes has yet been obtained.

Analysis of species in the other major line of bony fishes (subclass Actinopterygii, including chondrosteans, holosteans, and teleosts) reveals the presence of three genes encoding LDH subunits in all major groups studied (see Table 1). We found that the most primitive vertebrate clearly demonstrating the three genes for LDH—A, B, and C—is the sturgeon (see Fig. 2d). Virtually all species of bony fishes in the subclass Actinopterygii possess the C gene for LDH. The variation in both the structure and the regulation of this gene, as evidenced in these organisms, provides an informative picture of the evolution of a gene. An examination of the isozyme patterns of the sturgeon, paddlefish, and bowfin, each representing a primitive family, reveals the existence of many isozymes resulting from the polymerization of these three different subunits of LDH. All of these fishes exhibit a predominance of the A_4 isozyme in white skeletal muscle, although the LDH A gene is active in other tissues as well. The B gene functions predominantly in heart muscle and in red skeletal muscle, and also exhibits substantial activity in most other tissues. The C gene is also active in nearly every tissue and, significantly, the pattern of relative activity follows approximately that of the B gene, not the A.

We conclude from this tissue specificity of expression and from immunochemical, kinetic, and thermal stability data, that the C gene probably arose by duplication of the B, rather than the A gene. Immediately after duplication, the C gene, at that time indistinguishable from the B, must have been regulated in perfect register with the B gene and thus expressed to the same extent in the same tissues. Later, accumulation of mutations in the B and C genes would gradually have resulted in their divergence. This process is evident from an examination of the isozyme patterns (Table 1) of the sturgeon, the paddlefish, and the bowfin. The electrophoretic mobility and, thus, net charge of the C_4 isozyme varies from the most cathodal (sturgeon) to the most anodal (bowfin) of the LDH isozymes of these species. Thus, great variation in relative net charge is exhibited by the C_4 isozymes of these three primitive bony fishes. We know also, from an examination of mutants at the B locus in several vertebrates, that the mobility of this isozyme can be changed greatly by even single amino acid substitutions. Only a few changes in amino acid composition would be required to bring about the divergence in electrophoretic mobility evident between the B and C polypeptides in the zymograms of these three primitive fishes. Such changes in electrophoretic mobility are, of course, only a rough but nevertheless informative indicator of changes in the structure of the enzyme.

Some changes in enzyme structure would surely generate altered kinetic properties. In fact, measurements of the kinetic properties of the C_4 polypeptide, as purified from the tissues of several species of teleosts, do show that this isozyme is distinct from both the A_4 and B_4 isozymes. As the catalytic and other properties of the C_4 isozyme diverged from those of the B_4 during evolution, a metabolic situation was created in which selection for changes in tissue specificity of expression could occur. That is, the C_4 isozyme of LDH, with newly altered properties as compared with the ancestral B_4 isozyme, would occasionally be more advantageous in one specialized cell type as compared to another. Thus, an opportunity would be created for selective pressures to alter the regulation of the C gene so that its new metabolic characteristics could be exploited in those cells for which they were advantageous and suppressed in those cells for which they were not advantageous or were even dele-

terious. By this procedure, changes in expression of genes become possible as genes diverge in structure and their polypeptides acquire new properties. A study of the zymograms of the primitive bony fishes shows that the patterns of B and C activity are not identical, some evolutionary divergence having already occurred, but both genes are similar in tissue expression as contrasted to the expression of the A gene.

Proceeding up the evolutionary scale to the teleosts, we find again that the most primitive representatives express the C gene in a great many tissues and in a pattern that is similar to that of the B gene. As evolutionary specialization occurs, the function of the C gene is progressively restricted in an ever more specific fashion. For example, in primitive species such as the bonefish, purplemouth moray (Fig. 2c), American eel, butterfly fish, and the elephant nose (distributed in two major groups of primitive teleosts, the Elopomorpha and Osteoglossomorpha), the expression of the C gene is somewhat more restricted than in the nonteleosts (Table 1). Thus, although C subunits are present in many tissues they predominate in relatively few. The tissues in which the C subunits do predominate are quite varied in these fishes and include kidney, spleen, gills, and brain. In addition, some physical properties of the C subunits, for example, net charge, are highly varied in these fishes. Electrophoretic migration varies from slightly cathodal in some species to extremely anodal in others. This is in contrast to the relatively stable structure and tissue expression of both the A and B genes. It is fair to conclude from these data that the C gene at this intermediate evolutionary level is not yet highly specialized in either structure or regulation, although more so than in the nonteleosts discussed earlier.

In contrast to the general properties of the C gene in primitive bony fishes, the C gene is much more specialized in the advanced teleosts. Surprisingly, this specialization in both structure and regulation has taken either of two major directions in accord with the evolutionary history of each particular order or family of fishes. As revealed by Table 1, by far the majority of species examined express an eye-band C_4 isozyme (see Fig. 2f), while a few advanced groups possess a liver-band C_4 (Fig. 2e). Thus, among the Osteichthyes the general trend in evolution is clearly for specialization of the C gene and restriction of its function to neural tissues or, for a few fish, to the liver. The expression of the C gene predominantly in the liver is particularly characteristic for certain species in the orders Cypriniformes and Gadiformes, although within both of these orders some species have a C gene specialized for function in the eye rather than in the liver. We know that the kinetic properties of the C_4 tetramer are different from those of the A_4 and B_4 isozymes, but we do not yet have any insight into why the different kinetic properties are advantageous for the eye on the one hand, or for the liver on the other.

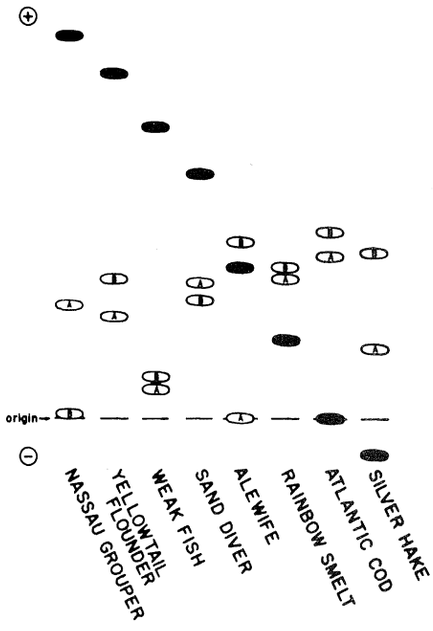


Fig. 3. Comparative electrophoretic mobilities of the C_4 isozymes of selected teleost fishes. The A_4 and B_4 homopolymers are indicated by the letters *A* and *B*, respectively. The C_4 isozymes are represented by the solid black bands. It is clear that the net charge of the C_4 isozyme is very different (from highly anodal in many species to slightly cathodal in a few) in different species of teleost fishes. (The fishes are not arranged in phylogenetic order.)

Liver specificity of C gene function observed in some of the Cypriniformes and Gadiformes is correlated with a relatively cathodal mobility (greater positive net charge) of the C_4 isozyme. In nearly all of the other teleosts, in which the C gene functions primarily in the eye, the mobility of the isozyme is markedly anodal. No clear rationale is now evident for this correlation of tissue specificity and net charge. Perhaps the net charge assigns the isozyme to a particular part of the cell. However, an overview of all of the fish examined in this study does make clear the absence of any rigid correlation between tissue specificity and electrophoretic mobility, that is, net charge on the isozyme (Table 1).

In general, there is an association of both structural and regulatory properties of this C gene such that the eye-band form tends to be highly anodal while the liver-band form is nearly always cathodal (Fig. 2). However, the above generalization is based only on frequency of occurrence. Figure 3 reveals that the net charge on the C subunit can vary over the complete electrophoretic range when examples are considered from a large number and variety of species. This virtually continuous range of variation in structure is paralleled by a similar continuous variation in regulation of the activity of the C gene. These data are summarized in Table 1. As expected, those species of intermediate evolutionary position exhibit somewhat variable C gene structure and regulation while the most advanced groups generally exhibit highly specialized and less variable characteristics of the C gene. These results present an evolutionary history of the LDH genes themselves, especially the C gene, and, as such, trace a path from the origin of the C by duplication of the B gene through evolutionary divergence to the eventual acquisition of unique and specialized structure, function, and regulation. A general outline of the evolutionary origin and divergence of vertebrate LDH's, as observed in fishes, is presented in Fig. 4.

Evolution of Recently Duplicated Genes

In our attempt to analyze the evolution of genes, we have found the Salmonidae, a family of fishes that includes the trouts and salmons, to be deserving of special attention. Members of this family have been shown to possess "extra" structural genes (more than those of diploid fishes) encoding various enzymes. A rather large body of information now supports the hypothesis that these fishes are essentially tetraploid. Presumably, their ancestors became tetraploid at least 20 million years ago and since that time evolution has been acting to bring about an effective "diploidization." On the basis of karyotype and DNA content (*2, 7, 78–80*), as well as the multiplicity and complexity of isozyme patterns (*47, 78, 81, 82*), it seems clear that salmonid fishes do indeed possess much more genetic information than other families in the order Salmoniformes, and indeed more than most other teleosts. Given that the genomes of these fishes have recently duplicated one may then ask what has been the fate of such recently duplicated structural genes? As outlined above, immediately following gene duplication, both gene copies would be structurally identical and regulated together. Subsequent evolutionary divergence would change both the structure and the regulation of each of these formerly identical genes.

Recent investigation of the cytoplasmic malate dehydrogenase (MDH; E.C. 1.1.1.37) of salmonid fishes has suggested

that the genes encoding this enzyme may indeed be at a very early stage of divergent evolution. Superficial observation of the MDH patterns of salmonids reveals that these fishes generally exhibit the same number and variety of isozymes as do diploid fishes. At this level of inquiry, there is no suggestion of double doses of genes coding for MDH subunits. However, detailed quantitative analysis of isozyme distributions in fishes heterozygous at the MDH loci has revealed the presence of "hidden" duplicated MDH genes (*83*). These data support the conclusion that the genes encoding MDH subunits have been duplicated in the "tetraploidization" of these fishes. However, except for allelic variants, the pairs of duplicated genes have not yet diverged sufficiently in structure to produce distinguishable products. Gene duplication has evidently occurred, while structural divergence (as indicated by electrophoretic mobility) has not.

The complexity of many enzyme systems in salmonids can be explained by invoking the presence of "additional" genes for these enzymes. This seems particularly true for the multitude of LDH isozymes. Both the A and B genes have been duplicated in these fishes. The smelts (family Osmeridae), closely related to the Salmonidae, are diploid and these fishes serve as a useful reference point in interpreting the more complicated LDH arrays of the salmonids. It is clear that smelt possess the A, B, and C genes for LDH (Table 1) and that the expression of the C gene is predominant in the eye (eye-band). The A and B subunits of LDH do not interact to form heteropolymers in smelt and, thus, these teleosts are "two-isozyme" fishes with regard to A-B tetramers. Only homopolymers (A_4 and B_4) are formed. Knowing this pattern, one can easily interpret the multiple isozymes of salmonids (Fig. 5 left). Duplicated A subunits, A^1 and A^2, polymerize randomly with one another to give all five possible A subunit-containing tetramers. The same is true for the B^1 and B^2 subunits, but as with the smelt, the A and B type subunits apparently do not interact to form heteropolymers. We have found no satisfactory evidence for duplicated C genes, although such a duplication should have occurred because the C gene existed long before the tetraploidization of these fishes. It is possible that, though duplicated, the two copies of the C gene, like those of the MDH genes, have not yet diverged sufficiently to be detected. Alternatively, one of the duplicated copies may have been silenced or lost during the ongoing evolution of these fishes.

Whatever the situation concerning the LDH C gene may be, it is the A and B genes of these salmonids that provide additional insight into gene evolution. In most tissues the expression of the duplicated genes (A^1 and A^2, B^1 and B^2) is virtually the same for each member of the pair. Note particularly in Fig. 5 left that the duplicated A genes are equally expressed in skeletal muscle tissue, giving rise to a binomial distribution of A^1-A^2 tetramers. However, in certain tissues, the regulation of function is clearly different (*39, 47, 82*). Despite the obvious similarities between these recently duplicated genes (and their polypeptide products), regulatory mechanisms are able to distinguish them. In the liver of the brown trout, for example (see Fig. 5 left), the B^1 subunits predominate, while in heart muscle the opposite is true, namely B^2 subunits predominate. In other tissues, such as the brain, both genes function more or less equally. However, in the brook trout (Fig. 5 right) the pattern of activation of B^1 and B^2 genes in heart muscle and liver contrasts sharply with that of the brown trout. Our nomenclature (*34*) designates the most anodal B polypeptide in each species as B^1, but tissue distribution and function indicate that the B^1 of brown trout is homologous to the B^2 of brook trout. Regardless of the method of designating these genes, it is evident that the duplicate B gene with the highest net negative charge predominates in the liver of the brown trout but not in the heart, and just the reverse is true for the brook trout. The significance of this observation is to indicate once again that the electrophoretic mobility, reflecting the net charge on the surface of the molecule, is not a critical enzymatic characteristic, although the net

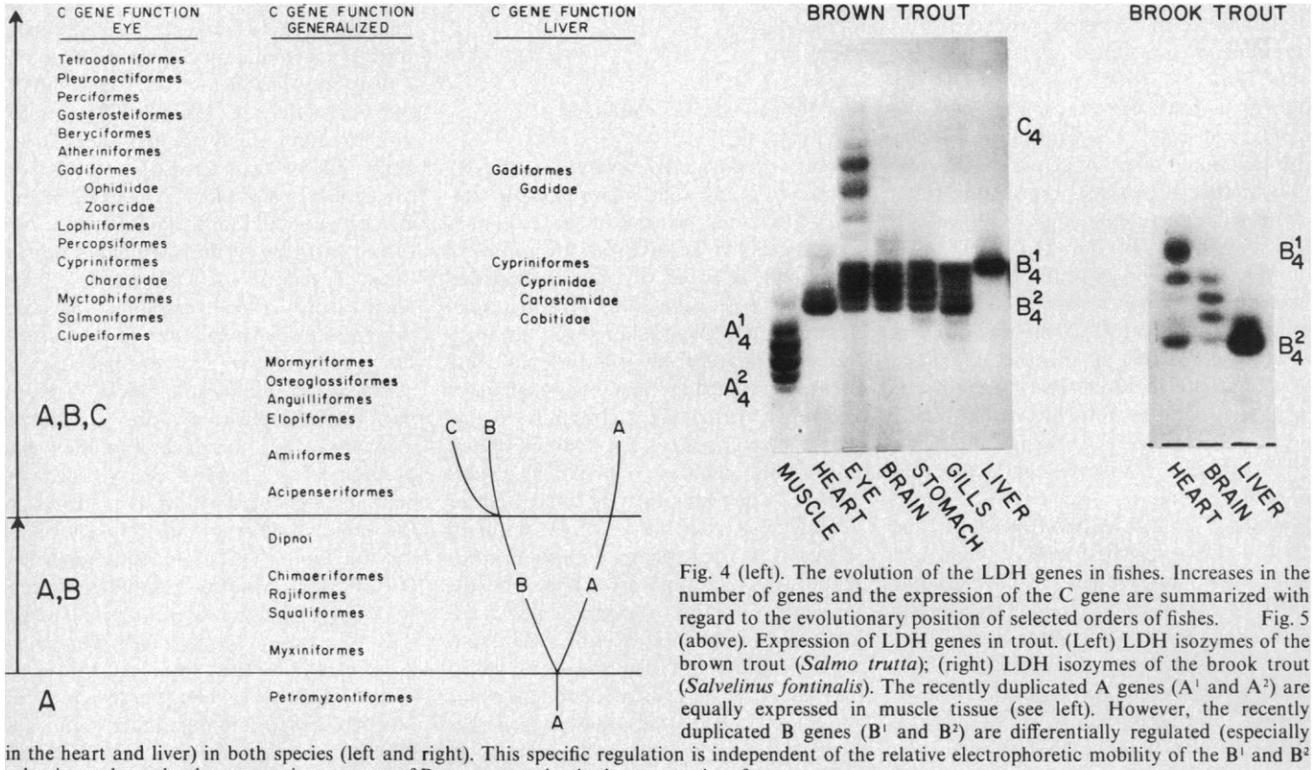


Fig. 4 (left). The evolution of the LDH genes in fishes. Increases in the number of genes and the expression of the C gene are summarized with regard to the evolutionary position of selected orders of fishes. Fig. 5 (above). Expression of LDH genes in trout. (Left) LDH isozymes of the brown trout (*Salmo trutta*); (right) LDH isozymes of the brook trout (*Salvelinus fontinalis*). The recently duplicated A genes (A^1 and A^2) are equally expressed in muscle tissue (see left). However, the recently duplicated B genes (B^1 and B^2) are differentially regulated (especially in the heart and liver) in both species (left and right). This specific regulation is independent of the relative electrophoretic mobility of the B^1 and B^2 subunits as shown by the contrasting patterns of B gene expression in the two species of trout.

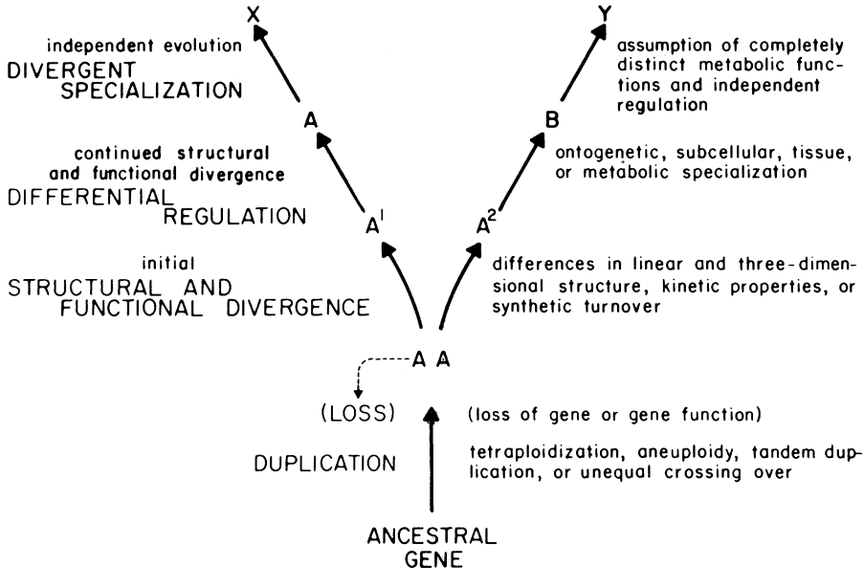


Fig. 6. Generalized scheme of gene evolution describing the evolution of structure, function, and regulation.

charge is probably significant in the general adaptive integration of the enzyme molecule into the metabolic machinery of the cell.

Evidently in these trout, changes in gene regulation have developed faster than changes in the basic structure of the duplicated B genes. We believe that the obvious differences in tissue specificity of gene expression in these trout reflect advantageous differences in the metabolic properties of the slightly divergent polypeptides.

Tetraploids are not restricted to the Salmonidae; many species in the order Cypriniformes also appear to be tetraploid (*7, 70, 77, 80, 84*). Investigation of LDH patterns (and other enzymes as well) indicates duplicated genes. However, as is the case for salmonid fishes, not all enzymes exhibit the number of isozymes expected to result from a complete doubling of the genome. Possibly many of the duplicated genes have not yet diverged sufficiently to be detected. They would remain "hidden" until mutated. Alternatively, duplicated DNA may have been lost or silenced. A gradual loss of genetic material seems to characterize the evolution of organisms (especially fishes) after large increases in DNA have occurred (*80*). Duplicate copies of genes should be somewhat freed from the constraints of natural selection since one copy should suffice for the needs of the cell. A duplicated copy might readily become nonfunctional because of deleterious mutations, or even be lost by unequal crossing-over or some other mechanism, without adversely affecting the organism. In this regard, the recent report of the possible existence of a "null" allele for one of the duplicated LDH B loci of the carp, a cypriniform fish, is especially suggestive (*85*). Populations of these carp exhibit a polymorphism for an "allele" which results in the absence of activity of a particular LDH subunit, the B^1 subunit. Similar results have recently been reported for the LDH B^1 gene of the goldfish, a closely related species (*86*). Although this evidence is somewhat tentative, these observations conform closely to the expectation that some duplicated genes will be lost during evolution and apparently provide a specific example of a transient intermediate state in this process.

The Process of Gene Evolution

Having considered several specific examples of gene evolution, we can now summarize essential features of this evolutionary process. As we have seen, the evolution of a gene is based on two fundamental processes: first, changes in the structure of a gene and, second, changes in its regulation. The nature and progression of these events is outlined in Fig. 6. Although genes evolve whether duplicated or not (for example, cytochrome c), the addition of new information requires duplication. In the complex organisms existing today and for a long time into the past, the primary source of new genetic material (new information) must be duplicated copies of existing genes, since the creation of a new useful gene seems impossible. Before gene duplication, there exists what might be called the "ancestral gene." Among the genes for vertebrate LDH's, this ancestral gene apparently resembled the contemporary A gene. The restricted repertory of LDH isozymes in the lamprey—only one A-like gene is evident—can be considered a living example of this primitive or ancestral state. Gene duplication, of course, generates the material upon which subsequent evolution will work (*7, 8*); the mechanisms for achieving such duplication are manifold and have been discussed at length elsewhere (*2, 7, 20, 87*). Basically, these mechanisms involve duplication of the entire genome (allo- and autotetraploidization), duplication of one or more chromosomes (aneuploidy), or duplication of a single gene (linear replication or unequal crossing-over). At first the duplicated genes will, in general, be identical. However, incomplete or excessive linear replication could lead to a different nucleotide sequence (either shorter or longer than the original), or unequal crossing-over in an organism heterozygous for two different alleles at the locus in question would result in instant differences between the two duplicated genes. Although each of these possibilities may have occurred, the major course of gene evolution seems to involve gene duplication that generates initially two identical copies of the ancestral gene. Since the two recently duplicated genes would have identical structures they should be expressed equally. A well-documented example of duplicated genes in this transient state of identity is provided by the duplicated MDH genes of salmonid fishes (*83*).

Following gene duplication there is a period of structural divergence (with attendant functional divergence) brought about by the accumulation of mutations in the structural genes themselves. Thus, initially identical duplicates become different. Examples of this slow accumulation of structural differences are clearly provided by the duplicate LDH loci (A^1 and A^2, B^1 and B^2) of salmonid and some cypriniform fishes. In many of these instances the duplicated genes experience more or less identical regulation even though the structural genes themselves are somewhat different. In addition to the duplication of complete genes or whole blocks of genes, partial gene duplication represents another mechanism by which gene evolution may proceed. The importance of such incomplete gene duplication is indicated by the large number of proteins known to contain internally homologous sequences (*88*). Further evolution promotes the continued accumulation of structural changes that differentiate the originally duplicated genes as well as their products. In concert with this continued change in structure and function, changes in regulation occur. The genetic apparatus begins to distinguish these once identical genes and as a result

the genes become differentially expressed. This stage of evolutionary divergence is exemplified by the LDH A, B, and C genes of fishes and other vertebrates.

During the process of gene evolution, changes in the modulation of effective gene expression can occur not only by controlling the level of genetic transcription, but also by altering the genetic structure of the protein product so that the protein acquires regulatory properties. The activities of such regulatory enzymes are modulated as a result of the binding of certain metabolic intermediates to the enzyme molecule. This modulation of enzymatic activity permits extremely rapid biochemical accommodation to changing metabolite levels as well as a delicate balancing of catalytic activity. Most regulatory enzymes respond to positive and negative modulators that are structurally similar to the substrates or coenzymes, or both, that are normally involved in the enzymatic reaction. For this reason it is tempting to speculate that partial or complete duplications of genes encoding enzyme subunits possessing catalytic activity may have provided the raw material from which these often complex regulatory enzymes have evolved. Indeed, recent studies of the partial amino acid sequence of glutamate dehydrogenase (GDH, E.C. 1.4.1.3, a regulatory enzyme from the liver of vertebrates) suggest that each of the six identical subunits making up this protein may contain internally homologous sequences, one involved in the catalytic activity of the enzyme, and the second serving only as a regulatory site for the binding of coenzyme (*89*). Thus, it seems that this regulatory enzyme may have evolved from a simpler nonregulatory enzyme as the result of a partial gene duplication conferring two binding sites on each subunit, one of which retained its enzymatic nature while the other evolved to serve a strictly regulatory function. In a similar way it seems likely that the regulatory subunits of many of the more complex enzymes, which are made up of both catalytic subunits and regulatory subunits (without catalytic capacity), may have evolved through partial or complete duplications of the gene encoding the original catalytic subunits. This evolution would have been characterized by the persistence of intersubunit binding sites which allow the continued association of the two types of subunits and by the loss of catalytic activity as the regulatory function was acquired.

The final stage of gene evolution appears when the originally identical genes become independent in both function and regulation. The LDH C gene of mammals and birds with its broad substrate specificity (for several α-hydroxy acids) and unique tissue expression (only primary spermatocytes) and the "steroid" and "ethanol" subunits of horse liver alcohol dehydrogenase (*90*) are approaching this independence. The many related dehydrogenases [for example, alcohol dehydrogenase (E.C. 1.1.1.1.), MDH, LDH, GDH, and glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12)] have already achieved this independence (*18*). Indeed, recent experiments with bacteria indicate that under certain conditions this evolution of changed substrate specificity can be a very rapid process (*9*). It should be pointed out that at each step in this process, the evolving gene is affected by mutation, drift, and natural selection not only as these processes transform the gene into a new and different gene, but also as they may result in the loss or "extinction" of the gene (*91*). The suggestion of "null" alleles for the LDH B^1 subunit of some cyprinid fishes, the well-documented null alleles of other eukaryotes (*92*), and the often observed inactive proteins of bacterial systems reflect the loss of gene expression. The retention of such inactive nucleotide sequences during subsequent evolution may account for some of the "excess" or "nonsense" DNA characteristic of the genomes of higher organisms (*2, 93*). Thus, just as genes can be made to speak new languages, they can also be silenced during the evolutionary process. It should be remembered when analyzing the evolution of genes and proteins, just as when analyzing the evolution of organisms, that the survivors today represent only the successes and not the failures.

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94. Abbreviations: EBT, ethylenediaminetetraacetate borate tris(hydroxymethyl)aminomethane; PMS, phenazine methosulfate; NBT, nitroblue tetrazolium.

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