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TUTORIAL T6

Protein Sequence Comparison and Protein Evolution

(William R Pearson)

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Protein sequence comparison and Protein evolution

ISMB95 Tutorial

William R. Pearson*

Department of Biochemistry,
Jordan Hall,#440
University of Virginia, Charlottesville, VA 22908, USA

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*FAX: (804) 924-5069; email: wrp@virginia.EDU

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1 Introduction

The concurrent development of molecular cloning techniques, DNA sequencing methods, rapid sequence comparison algorithms, and computer workstations has revolutionized the role of biological sequence comparison in molecular biology. As a result, the role of protein sequence data in molecular biology and biochemistry has dramatically changed. Twenty-five years ago, protein sequence determination was usually one of the last steps in the characterization of a protein. Now the process is reversed, so that it is common to clone and sequence a

gene of biological interest–e.g., one that is induced by serum stimulation, or a developmental change, or a chromosomal rearrangement associated with a disease. This is the fundamental premise of the human genome project—that one can first sequence all the genes in an organism and then infer their function by sequence analysis.

Today, the most powerful method for inferring the biological function of a gene (or the protein that it encodes) is by sequence similarity searching on protein and DNA sequence databases. With the development of rapid methods for sequence comparison, both with heuristic algorithms and powerful parallel computers, discoveries based solely on sequence homology have become routine. One of the more dramatic discoveries was the identification of a new tumor suppressor gene in humans that is related to yeast and *E. coli* DNA repair enzymes. This discovery, the result of a similarity search, both told the investigators that they had identified the appropriate gene and demonstrated clearly the nature of the oncogenic mutation. As entire genomes from bacteria, yeast, and simple eukaryotes become available, protein sequence comparison will become an even more powerful tool for understanding biological function.

Protein sequence comparison is our most powerful tool for characterizing protein sequences because of the enormous amount of information that is preserved throughout the evolutionary process. For many protein sequences, an evolutionary history can be traced back 1–2 billion years. Proteins that share a common ancestor are called *homologous*. Sequence comparison is most informative when it detects *homologous* proteins. Homologous proteins always share a common three-dimensional folding structure and they often share common active sites or binding domains. Frequently homologous proteins share common functions, but sometimes they do not. Our ability to characterize the biological properties of a protein based on sequence data alone stems almost exclusively from properties conserved through evolutionary time. Predictions of common properties for non-homologous proteins—similarities that have arisen by convergence— are much less reliable.

This tutorial examines how the information conserved during the evolution of a protein molecule can be used to infer reliably *homology*, and thus a shared protein fold and possibly a shared active site or function. We will start by reviewing a geological/evolutionary time scale. Many protein sequences can be used to infer reliably events that happened more than a billion years ago. Remarkably, some protein sequences change so slowly that they could be used to "date" events that took place more than 5 billion years ago, had the proteins existed. Next we will look at the evolution of several protein families. During the tutorial, these families will be used to demonstrate that homologous protein ancestry can be inferred with confidence. We will also examine different modes of protein evolution and consider some hypotheses that have been presented to explain the very earliest events in protein evolution.

The next part of the tutorial will examine the technical aspects of protein sequence comparison. Both optimal and heuristic algorithms and their associated parameters that are used to characterize protein sequence similarities are discussed. Perhaps more importantly, we will survey the statistics of local similarity scores, and how these statistics can both be used to improved the selectivity of a search and to evaluate the significance of a match.

We will then examine distantly related members of three protein families, the serine proteases, the glutathione transferases, and the G-protein-coupled receptors (GCRs). The serine

proteases are used to emphasize that even when a highly conserved motif is found throughout a family, similarity extends over a much longer region. The glutathione transferases and GCRs are very diverse families whose members frequently do not share significant pairwise similarity. The relative strengths of strategies to characterize such relationships will be examined.

Finally, we will discuss how sequence similarity can be used to examine internal repeated or mosaic structures in proteins. Such repeated structures can arise from either divergence—calmodulin EF-hand repeats and EGF-domains—or convergence—tropomyosin and transcription factor coiled-coil.

This tutorial is directed towards examining protein evolution. Most of the algorithms and methods that are applied to protein evolution can be used with DNA sequences as well. However, in general, DNA sequence comparisons are far far less informative than protein sequence comparisons (see Fig. 8). DNA sequences that do not encode proteins or structural RNAs (e.g. ribosomal RNAs) diverge very rapidly, so that it is usually difficult to detect reliably non-coding DNA sequence homologies for sequences that diverged more than 200 million years ago. In contrast, even the most rapidly changing protein sequences can detect sequences that are 200 million years old; typically protein sequence comparisons detect sequences that diverged 1 billion years ago. Thus, the most important lesson from this tutorial is, when searching sequence databases for homologous sequences, to use protein sequences whenever possible.

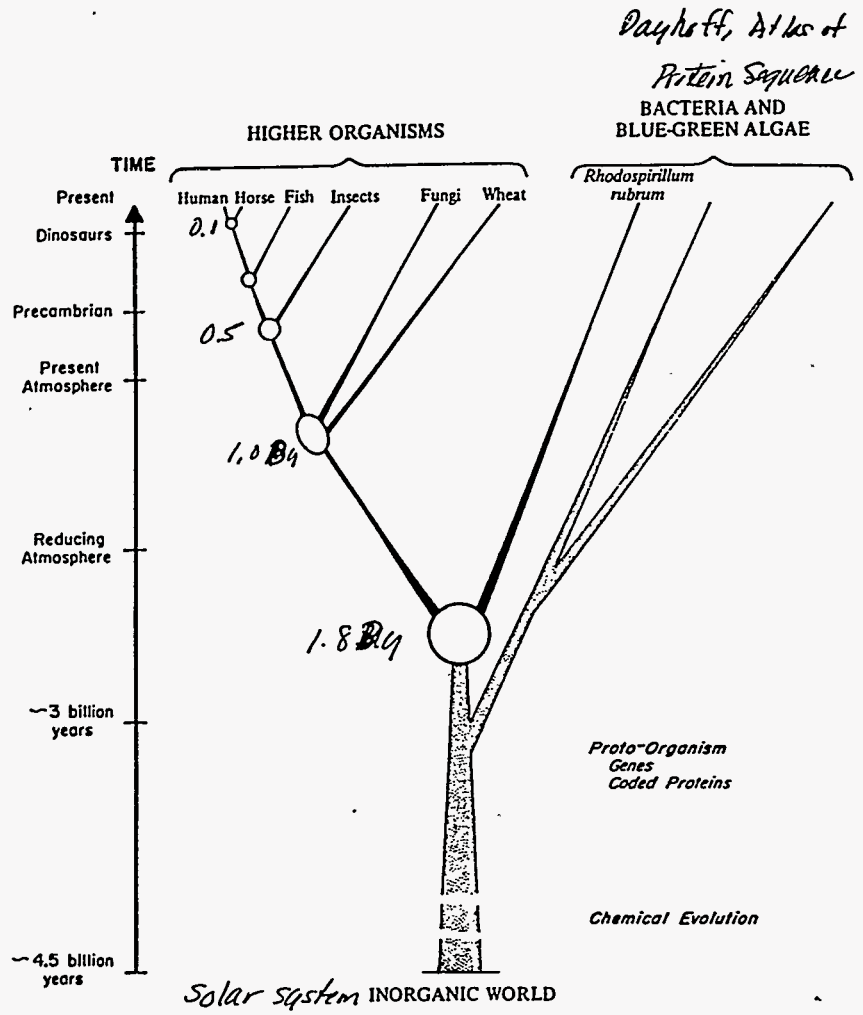
1.1 Evolutionary time scales

When we search for *homologous* proteins, we are trying to identify proteins that shared a common ancestor in the past. Fig. 1 shows a general evolutionary tree that reaches back to the beginning of the earth's history. The goal of protein sequence comparison is to take a protein sequence, for example from a human chromosome, and search a protein database to find *homologous* sequences, often from very divergent organisms. Thus, if the similarity search produces significant matches with a protein found in yeast, then an ancestral protein must have existed in an organism at least 1 billion years ago and that the descendants of that organism preserved the sequence in modern day humans and yeast. Likewise, if a yeast protein is homologous to one found in *E. coli*, that sequence must have existed in 2 billion years ago in the primordial organism that gave rise to bacteria and fungi.

When we examine protein or DNA sequences, we are almost always studying modern (present day) sequences. Thus, it does not make any sense to say that a yeast or bacterial sequence is more primitive than a mammalian sequence; all sequences are contemporary. As we will see later, however, there are examples of sequences that are found only in vertebrates, or only in animals or plants but not both. Such sequences are less ancient than those found both in mammals and bacteria.

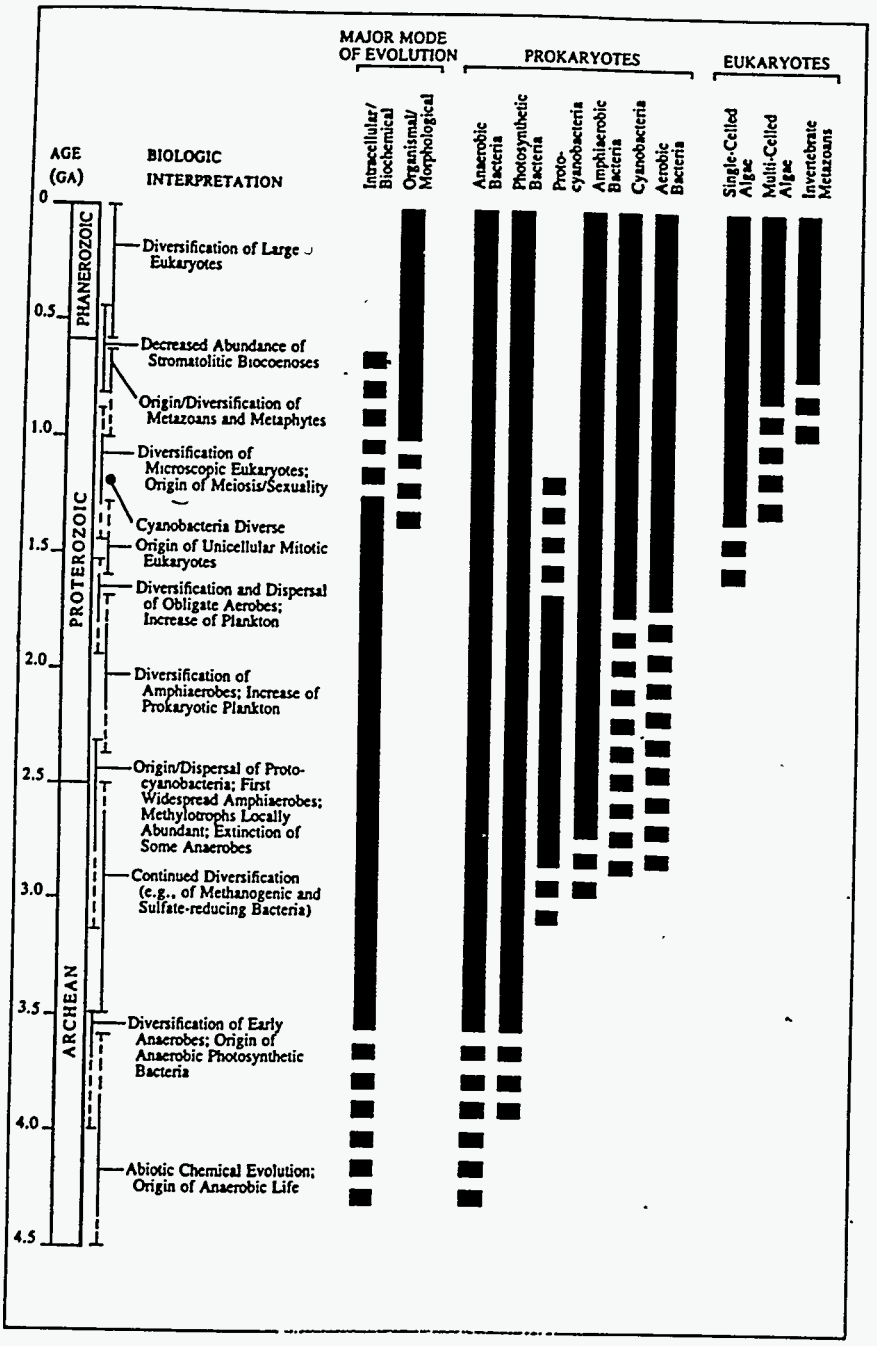
For organisms that diverged within the past 600 Mya, inferences about divergence times for modern organisms are taken from geological data; more ancient divergence times are inferred from extrapolations of evolutionary "clocks." Evolutionary clocks are based both on slowly changing protein sequences and on ribosomal RNA sequences; such divergence time

Figure 1: The tree of life



From Dayhoff *et al.*, 1978.

Figure 2: Geologic time scales



From Nei, 1987.

estimates require a rate of change that is constant on average. The oldest fossils are of prokaryotes in rocks about 2.5 billion years old; this geological age is consistent with that inferred from evolutionary divergence rates.

Table 1: Some Important dates in history

Origin of the universe	-10[a]	±2
Formation of the solar system	-4.6	±0.4
First self-replicating system	-3.5	±0.5
Prokaryotic-eukaryotic divergence	-1.8	±0.3
Plant-animal divergence	-1.0	
Invertebrate-vertebrate divergence	-0.5	
Mammalian radiation beginning	-0.1	

[a]Billions of years. From Doolittle *et al.*, 1986.

Table 2: Evolutionary Horizons

Protein	PAMs[a]/100 residues /10^8 years	Theoretical Lookback time[b]	Horizon
Pseudogenes	400	45[c]	Primates, Rodents
Fibrinopeptides	90	200	Mammalian Radiation
Lactalbumins	27	670	Vertebrates
Ribonucleases	21	850	Animals
Hemoglobins	12	1.5[d]	Plants/Animals
Acid Proteases	8	2.3	Prokayrotic/Eukarotic
Triosphosphate isomerase	3	6	Archaen
Glutamate dehydrogenase	1	18	

[a]PAMs, point accepted mutations. [b]Useful lookback time, 360 PAMs, 15% identity.
[c]Millions of years. [d]Billions of years. Adapted from Doolittle *et al.*, 1986

Table 1 summarizes some important milestones in evolutionary time, and, when considered with Table 2, gives a better perspective on the the evolutionary horizons provided by different protein families. The theoretical lookback times in Table 2 are based on the assumption that one can identify proteins that share about 20% sequence identity throughout their entire length. It will be clear from later examples that if two protein sequences share 25% identity across their lengths, they are homologous, and that in some cases, convincing evidence of common ancestry can be deduced from similarities as low as 20%. These lookback times can be confirmed in practice; for example, with sensitive sequence comparison

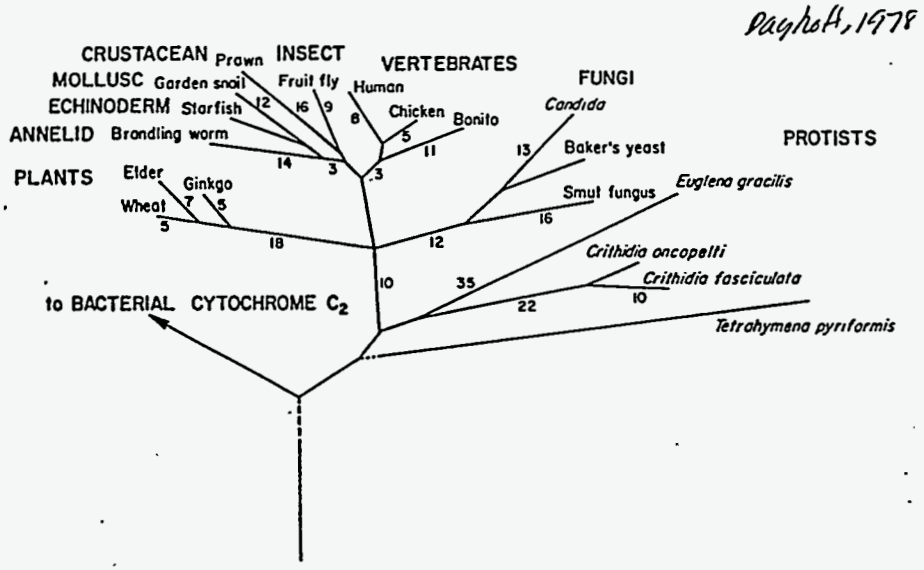
algorithms, significant similarity between plant and animal globins can be found.

1.2 Modes of Evolution

1.2.1 Conventional divergence from a common ancestor

Homologous sequences can be divided into two groups: (1) *orthologous* sequences — sequences that differ because they are found in different species; and (2) *paralogous* sequences — sequences that differ because of a gene duplication event. Fig. 3 shows an evolutionary tree for *orthologous* cytochrome 'c' sequences. The branching pattern, which reflects the differences between cytochrome 'c' sequences, matches the evolutionary relationships of the species that express the the proteins.

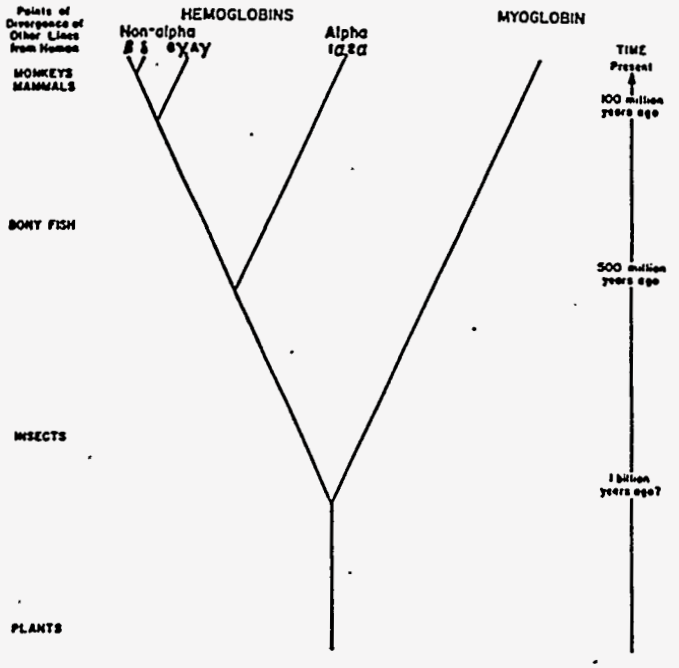
Figure 3: Orthologous sequences — The cytochrome 'c' family



Cytochrome 'c's comprise a family of orthologous proteins that are found in all organisms. This branch of the tree shows the number of differences between different eukaryotes. Thus, human and chicken cytochrome 'c', which diverged about 400 Mya, differ at 13 of 110 positions. The sequences on this tree are *orthologous* — two cytochrome 'c's are different because they are in different species.

In general, the organismal tree and the sequence tree will not match if the sequences are *paralogous*. Members of the globin oxygen binding protein family are both *orthologous* — they differ because of speciation — and *paralogous* — they differ because of gene duplications. Thus, human α-globin, mouse α-globin, and chicken α-globin are all orthologs, they differ because of the speciation events that gave rise to humans, rodents, and birds. Mouse β

Figure 4: Orthology and paralogy — The globin family



globin and human α globin are paralogous; they differ because of a gene duplication that created the α and β subunits some 600 Mya. An evolutionary tree based on human α, chicken α, and mouse β would imply that humans are more closely related to chickens than to mice. While such a mistake is unlikely in a well-studied family like the globins, it can be quite common in large, diverse, and poorly characterized families like the G-protein-coupled receptors (Fig. 22).

1.2.2 Sequence similarity and homology, the H^+ ATPase

Our first example of the significant sequence similarity shared by homologous proteins will use one of the chains of the H^+-ATPase, or proton-pump, used to convert energy to ATP in the mitochrondria and chloroplasts of aerobic organisms. Table 3 reports similarity scores and their statistical significance from a search of the PIR annotated protein sequence database (PIR1, release 44, March, 1995) using the human H^+-ATPase as a query sequence. There is excellent agreement between the expected and actual distributions of similarity scores. In this search, all of the library sequences related (homologous) to the query sequence obtained scores higher than any of the unrelated sequences. However, a number of unrelated sequences obtained very high scores; 10 of the 32 sequences with z-scores > 120 (7 standard deviations above the mean [1]) are not members of the H+-ATPase family.

[1]The z-scores plotted have a mean of 50 and a standard deviation of 10.

Figure 5: Searching with human ATP-ase, similarity scores

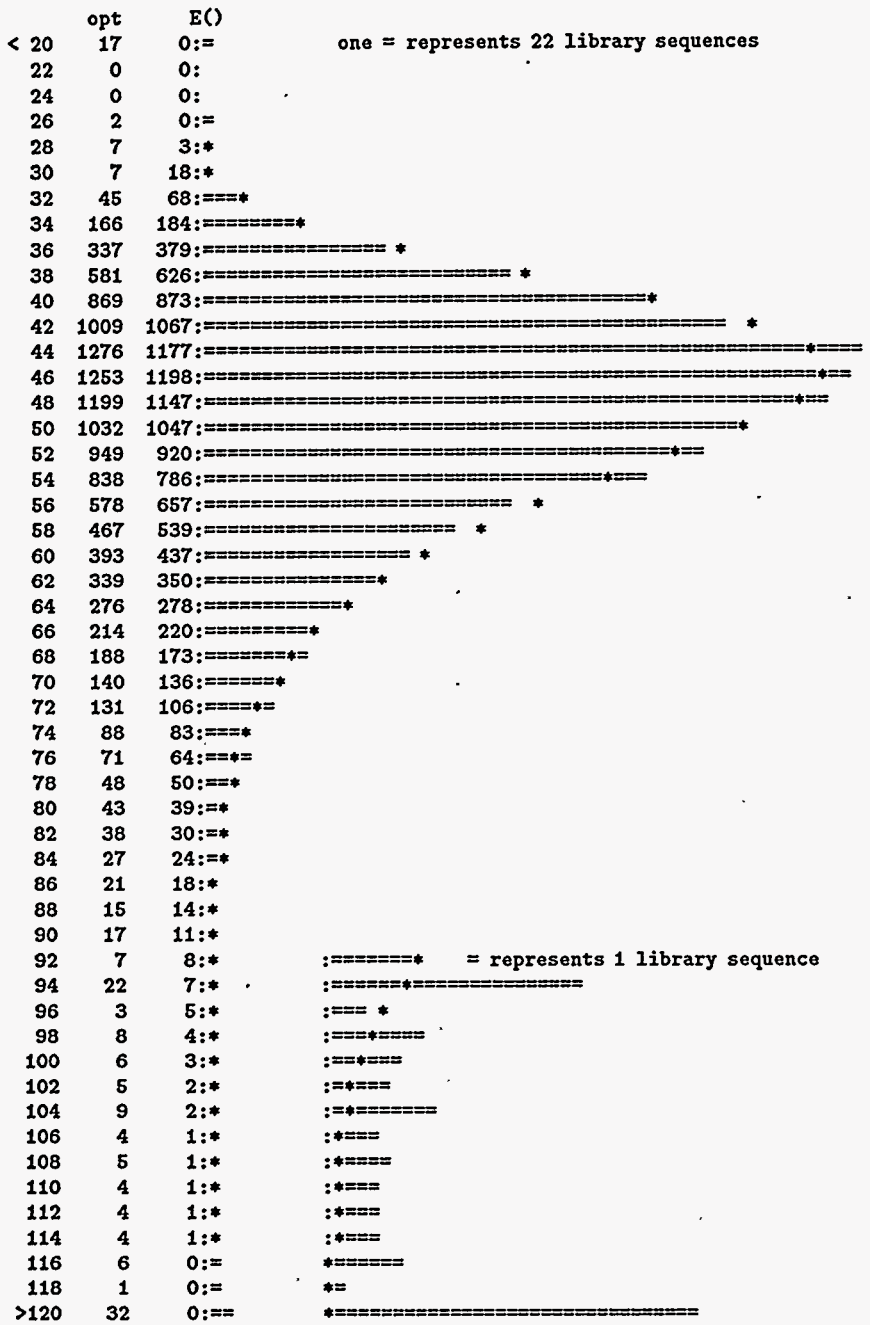


Fig. 5 shows the distribution of similarity scores between human H^+-ATPase (PIR entry PWHU6) and each protein sequence in the PIR1 (rel. 44) database. The '=' symbols in the histogram show the distribution of normalized similarity scores calculated during the search, thus, 393 sequences in the PIR1 library had scores of 60 or 61. The '*' symbols report the expected number of sequences with the indicated range of scores for a search of a database of this size, based on random chance. The basis for the statistical estimates will be discussed in section 3.

While Table 3 shows that all of the members of this family have siginificant similarity with the human enzyme, Fig. 6 gives a more realistic perspective of the family's evolutionary history by considering every possible pairwise alignment. When the *E. coli* enzyme is used to search the database for related H^+-ATPases, the ranking of the different sequences changes, but sequences distant from the *E. coli* sequence have more significant similarities than those distant from the human sequence.

The similarity scores in Figs. 5–7 where calculated using the Smith-Waterman algorithm, a method that guarantees to calculate the best (optimal) score between any two protein or DNA sequences, given a scoring matrix and gap penalties. Fig. 8 shows the PAM250 matrix, which was developed almost 20 years ago by Dayhoff and her colleagues (Dayhoff *et al.*, 1978). The PAM250 matrix, or modern versions such as the BLOSUM50 matrix used here, incorporates information about the likelihood that one amino-acid will be mutated into another over evolutionary time. Thus, changes that are very unlikely to occur in evolution, for example the substitution of the very small glycine residue for the very large tryptophan residue, are given large negative scores (−7 in Fig. 8), while conservative changes, such as the substitution of lysine by arginine (both have basic side chains), are given positive scores (+3). The scores for identical matches also vary in the PAM250 matrix, depending on whether the amino-acids are common (e.g. serine and methionine), and thus likely to be aligned by chance, or rare (e.g. cysteine and tryptophan). There is a well-developed statistical theory for substitution matrices (Altschul, 1991), which will be discussed in section 2.3.

For many protein families with a variety of divergence rates, the rate of change over evolutionary time is relatively constant (Fig. 9). These rates can be used to date the divergence events (e.g. plants and animals) that occurred more than 600 Mya and thus do not have a fossil record. However, different protein families diverge at different rates, so that, in general, the number of differences between a pair of sequences cannot be used to estimate the time the two sequences diverged. This is particularly true for paralogous sequences; once a sequence has duplicated, it may change very rapidly before selective pressure on its new function slows its rate of change. Thus, in Table 9 there are several members of growth hormone superfamily—growth hormone, sommatotropin, and prolactin—with different divergence rates.

1.2.3 Mosaic proteins

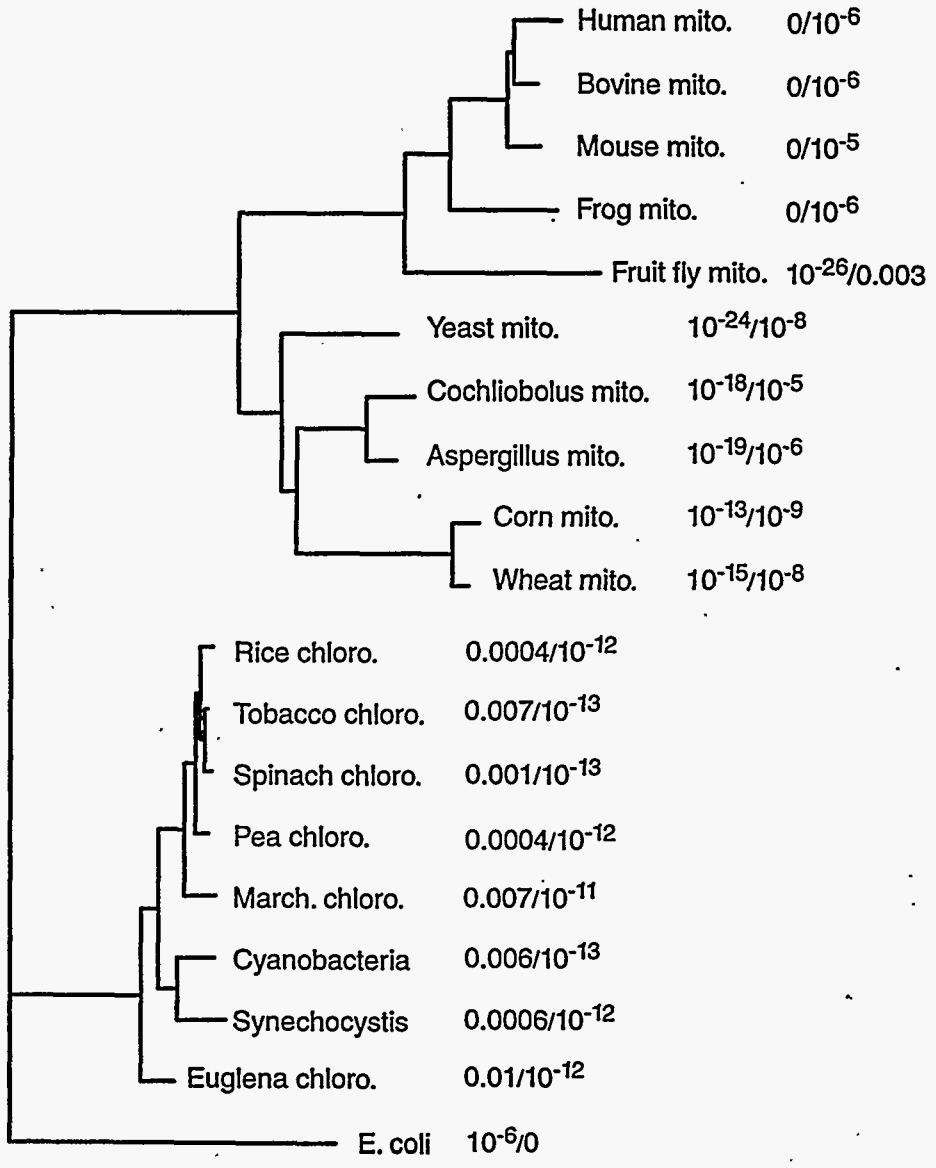
"Conventional" protein families, e.g. the globins, cytochrome 'c's, H^+-ATPases, in which protein sequences have diverged from a common ancestor in a direct fashion, typically with only modest changes in the length of the sequence, have been known for more than 30 years.

Table 3: Searching with human ATP-ase, high-scoring sequences

The best scores are:		s-w	z-score	E(12805)	%	len
PWHU6	H+-trans. ATP synth.—human mito.	1400	1767.8	0	100.0	226
PWBO6	H+-trans. ATP synth.—bovine mito.	1157	1460.9	0	77.9	226
PWMS6	H+-trans. ATP synth.—mouse mito.	1118	1411.6	0	75.7	226
PWXL6	H+-trans. ATP synth.—frog mito.	745	940.6	0	53.3	226
PWFF6Y	H+-trans. ATP synth.—fruit fly mito.	473	597.1	10^{-27}	37.8	222
PWFF6	H+-trans. ATP synth.—fruit fly mito.	471	594.6	10^{-26}	37.5	224
PWBY3	H+-trans. ATP synth.—yeast mito.	438	551.7	10^{-25}	36.2	232
PWAS6N	H+-trans. ATP synth.—aspergillus mito.	365	459.6	10^{-19}	30.4	230
PWKQ6	H+-trans. ATP synth.—Cochliobolus mito.	353	444.4	10^{-18}	31.3	214
PWWT6	H+-trans. ATP synth.—wheat mito.	309	385.4	10^{-15}	28.9	235
PWNT6M	H+-trans. ATP synth.—tobacco mito.	309	385.2	10^{-15}	28.3	233
PWZM6M	H+-trans. ATP synth.—corn mito.	283	355.0	10^{-15}	31.1	291
LWEC6	H+-trans. ATP synth.—E. coli	178	223.0	10^{-6}	23.3	236
LWRZ6	H+-trans. ATP synth.—rice chloro.	144	180.8	0.00037	24.2	231
PWPMA6	H+-trans. ATP synth.—pea chloro.	143	179.5	0.00044	25.0	232
PWYBAA	H+-trans. ATP synth.—Synechocystis	142	177.3	0.00058	26.5	170
PWSPA6	H+-trans. ATP synth.—spinach chloro.	138	173.2	0.00098	24.2	231
PWYCA6	H+-trans. ATP synth.—cyanobacteria	127	158.9	0.0062	26.3	167
LWNT6	H+-trans. ATP synth.—tobacco chloro.	126	158.1	0.0069	22.1	231
LWLV6	H+-trans. ATP synth.—Marchiantia chloro.	126	158.0	0.0069	24.0	167
PWEGAC	H+-trans. ATP synth.—Euglena chloro.	123	154.1	0.011	25.7	214
S17420	ubiquinol-cytochrome-c reductase	113	138.0	0.09	23.4	158
S17418	ubiquinol-cytochrome-c reductase	108	131.7	0.20	24.5	208
QXBO2M	NADH dehydrogenase (ubiquinone)	107	131.2	0.22	26.1	211
S17415	ubiquinol-cytochrome-c reductase	105	127.9	0.33	27.7	137
DNHUN2	NADH dehydrogenase (ubiquinone)	103	126.1	0.41	20.1	149
QRECAA	amino acid trans. protein—E. Coli	104	125.1	0.47	23.4	111
CBHU	ubiquinol-cytochrome-c reductase	102	124.1	0.53	26.8	205
S17419	ubiquinol-cytochrome-c reductase	101	122.9	0.63	23.4	158
S17407	ubiquinol-cytochrome-c reductase	99	120.3	0.87	23.6	140
QQBEN5	integral membrane protein—saimiriine herp	98	119.4	0.99	20.8	202

The horizontal line indicates the separation been the lowest scoring related sequences and the highest scoring unrelated sequence.

Figure 6: Phylogeny of H^+-ATPases



An evolutionary tree of H^+-ATPases (subunit 6). Sequences were aligned using the GCG PILEUP program, distances calculated using the GCG DISTANCES program, and the tree constructed using the Neighbor-Joining algorithsm (GCG GROWTREE). Expectation values from a search with the human H^+-ATPase (PWHU6, Table 3) and a search with the *E. coli* sequence are shown.

Figure 7: Searching with human ATPase, high-scoring sequences

```
LWEC6 H+-transporting ATP synthase (EC 3.6.1.34) protein - E. coli (271 aa)
 z-score: 223.0 Expect: 1.665e-06
Smith-Waterman score: 178;    23.3% identity in 236 aa overlap

                                    10        20        30        40        50        60
PWHU6                          MNEMLFASFIAPTILGLPAAVLIILFPPLLIPTSKYLINNRLITTQQWLIKLTSKQMMTMHNTKGRT
                                    :..  ..:::   ....::  .   ...  . ... :. . .: ... .. :.. :..
LWEC6  HLNNLQLDLRTFSLVDPQNPPATFWTINIDSMFFSVVLGL---LFLVLFRSVAKKATSG-VPGKFQTAIELVIGFVNGSVKDMYHGKSKL
          20        30        40        50          60        70        80        90       100

       70        80                  90           100       110       120       130
PWHU6  WSLMLVSLIIFIATTMLLGLLP---------HSF-------TPTTQLSMNLAMAIPLWAGTVIMGFRSKIKNALAHFLPQGTPTPL----
       . . .......   ::. :::          : .      .:.......:.::. ..   ... : :  .... :  . :  :.
LWEC6  IAPLALTIFVWVFLMNLMDLLPIDLLPYIAEHVLGLPALRVVPSADVNVTLSMALGVF---ILILFYSIKMKGIGGFTKELTLQPFNHWA
            110       120       130       140       150          160       170       180

       140       150       160       170       180       190       210       220
PWHU6  -IPMLVIIETISLLIQPMALAVRLTANITAGHLLMHLIGSATLAMSTINLPSTLIIFTILILLTILEIAVALIQAYVFTLLVSLYLHDNT
       ::. .:.: .::: .:..:..:: .:. ::.:.. ::..     :   :      :: :::.          .::..: .:. .::
LWEC6  FIPVNLILEGVSLLSKPVSLGLRLFGNMYAGELIFILIAGLLPWWSQWILNVPWAIFHILIIT---------LQAFIFMVLTIVYLSMASEEH
       190       200       210       220       230       240       250                 260       270

================

PWEGAC H+-transporting ATP synthase (EC 3.6.1.34) chain (251 aa)
 z-score: 154.1 Expect: 0.01133
Smith-Waterman score: 123;    25.7% identity in 214 aa overlap

                         10        20        30        40        50        60          70
PWHU6               MNEMLFASFIAPTILGLPAAVLIILFPPLLIPTSKYLINNRLITTQQWLIKLTSKQMMTMHNTK-GRT----WSLMLVSL
                                  .::: :  : : :.: :  . . ...: .:.:... .  . .:. :.    :  .. ...
PWEGAC IANVEVGQHFYWSILGFQIHGQVLINSWIVILIIGF--LSIYTTKNL--TLVPANKQIFIELVTEFITDISKTQIGEKEYSKWVPYIGTM
      20        30        40        50          60          70        80        90       100

       80         90             100       110       120       130       140       150
PWHU6  IIFIATTMLLG-LLPHSFT--PTTQL---SMNLAMAIPLWAGTVIMGFRSKI-KNALAHFLPQGTPTPLIPMLVIIETISLLIQPMALAV
       .:: ..:  : :.: ..   :. .:   .  ..  .  :   : .  : . . :..:..:     :::.. . :.: ..   .:..:.
PWEGAC FLFIFVSNWSGALIPWKIIELPNGELGAPTNDINTTAGLAILTSLAYFYAGLNKKGLTYFKKYVQPTPILLPINILEDFT---KPLSLSF
       110       120       130       140       150       160       170       180          190

     160       170       180       190       200       210       220
PWHU6  RLTANITAGHLLMHLIGSATLAMSTINLPSTLIIFTILILLTILEIAVALIQAYVFTLLVSLYLHDNT
       :: .:: : .:.. .. :         .:  ::. . ::.: ..   .. ::: .:. : . :.
PWEGAC RLFGNILADELVVAVLVSL--------VP--LIVPVPLIFLGLF---TSGIQALIFATLSGSYIGEAMEGHH
          200       210               220          230       240       250
```

Alignments of human H^+-ATPase with the *E. coli* homologue and a plant chloroplast homologue. Despite the considerable evolutionary distance (both sequences diverged at least 2 Bya), the pairs of sequence share more than 20% identity across almost their entire lengths. ':' symbols denote identities; '.' denote conservative substitutions. Searches were performed with the BLOSUM50 matrix and gap penalties of -12/-2.

Figure 8: The PAM250 matrix

Cys	12																				
Ser	0	2																			
Thr	-2	1	3																		
Pro	-1	1	0	6																	
Ala	-2	1	1	1	2																
Gly	-3	1	0	-1	1	5															
Asn	-4	1	0	-1	0	0	2														
Asp	-5	0	0	-1	0	1	2	4													
Glu	-5	0	0	-1	0	0	1	3	4												
Gln	-5	-1	-1	0	0	-1	1	2	2	4											
His	-3	-1	-1	0	-1	-2	2	1	1	3	6										
Arg	-4	0	-1	0	-2	-3	0	-1	-1	1	2	6									
Lys	-5	0	0	-1	-1	-2	1	0	0	1	0	3	5								
Met	-5	-2	-1	-2	-1	-3	-2	-3	-2	-1	-2	0	0	6							
Ile	-2	-1	0	-2	-1	-3	-2	-2	-2	-2	-2	-2	-2	2	5						
Leu	-6	-3	-2	-3	-2	-4	-3	-4	-3	-2	-2	-3	-3	4	2	6					
Val	-2	-1	0	-1	0	-1	-2	-2	-2	-2	-2	-2	-2	2	4	2	4				
Phe	-4	-3	-3	-5	-4	-5	-4	-6	-5	-5	-2	-4	-5	0	1	2	-1	9			
Tyr	0	-3	-3	-5	-3	-5	-2	-4	-4	-4	0	-4	-4	-2	-1	-1	-2	7	10		
Trp	-8	-2	-5	-6	-6	-7	-4	-7	-7	-5	-3	2	-3	-4	-5	-2	-6	0	0	17	
	C	S	T	P	A	G	N	D	E	Q	H	R	K	M	I	L	V	F	Y	W	

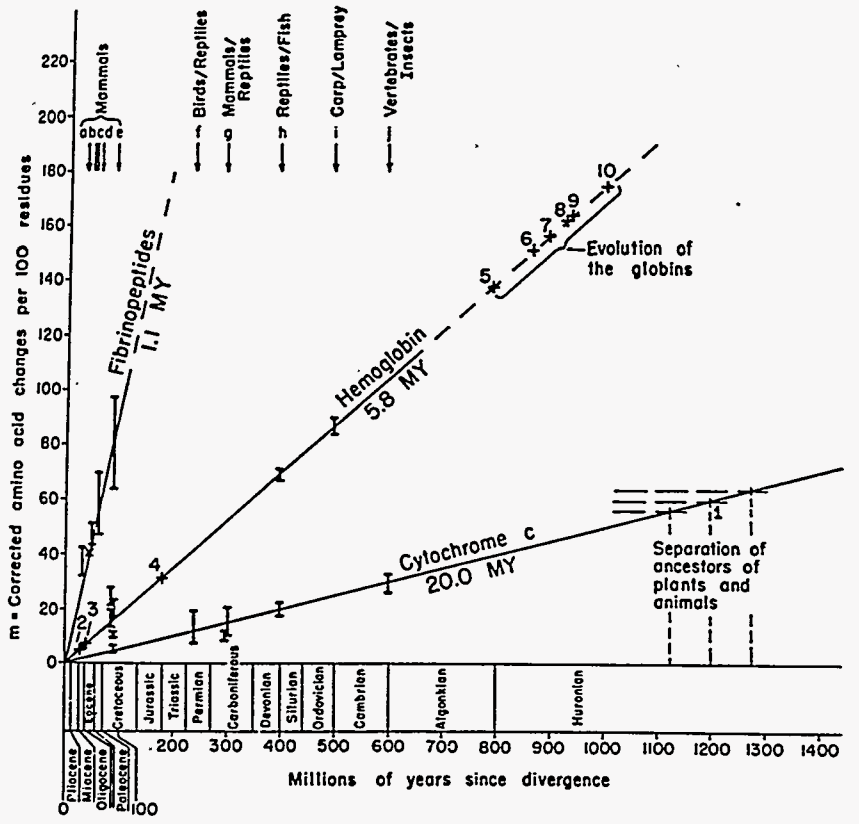
In the past 10 years, a more complex type of protein evolution has been observed—proteins that contain multiple domains from other proteins. These proteins have been called "mosaic" proteins; the domains are frequently inserted through a process called "exon shuffling." Table 7 lists a number of human proteins that are comprised of mosaic domains, but such proteins are not limited to mammals. Similar mosaic structures are common in DNA binding proteins, both in bacteria and eukaryotes. Fig. 11 shows the structures of some of the mosaic proteins in Table 7.

1.3 Introns Early/Late

The occurrence of mosaic proteins and the discovery of the "exon/intron" structure of genes in the late 1970's lead several investigators to suggest that the exon structure of genes reflected the construction of proteins from modular domains (Gilbert & Glynias, 1993). While acceptance of this proposal is quite widespread, it is based on very little data. There is no question that many modern mosaic proteins are constructed by a process of "exon-shuffling" whereby exons from other genes have been combined to build new structures. In addition, for some proteins exons are associated with well defined structural elements. The association of exons with structural elements may reflect and ancient construction of proteins from primordial exons. Alternatively, introns are also capable of invading genes; thus, the association of exons with structures may reflect modern invasions that are less disruptive when they occur between structural elements.

A recent test of the "introns" early hypothesis suggests there is little evidence to support the association of introns with structural boundaries (Stoltzfus *et al.*, 1994; Fig. 12). shows one of the figures from this paper, which is used to demonstrate a lack of correspondence

Figure 9: Rates of change in protein families



between several structural and the intron/exon boundaries.

1.4 DNA vs Protein comparison

While all of the comparison methods described below work on either protein or DNA sequences, one's ability to identify distantly related sequences is reduced dramatically when DNA sequences are used. Table 8 compares the statistical significance of the best similarity scores obtained in a search of the GenBank DNA sequence database using a mouse glutathione transferase cDNA clone with the significance of the same alignment in a search of the GenPept protein sequence database (GenPept is derived from GenBank by translating DNA sequences into the encoded protein sequences). Many DNA sequences encoding clearly related proteins, e.g. RABGSTB have similarity scores that are expected to occur several times by chance in a DNA database search. DNA sequences are far less informative, both because they lack the inherent biochemical information that is retained in the PAM250 substitution matrix, and because many changes in DNA sequences (third-base changes) do not change the encoded protein.

Differences in the performance of sequence comparison algorithms are insignificant com-

Table 4: Rates of change in protein families

Protein	*Rate*[a]	*Protein*	*Rate*
Fibrinopeptides	90	Thryrotropin beta chain	7.4
Growth hormone	37	Parathyrin	7.3
Ig kappa chain C region	37	Parvalbumin	7.0
Kappa casein	33	BPTI Protease inhibitors	6.2
Ig gamma chain C region	31	Trypsin	5.9
Lutropin beta chain	30	Melanotropin beta	5.6
Ig lambda chain C region	27	Alpha crystallin A chain	5.0
Complement C3a	27	Endorphin	4.8
Lactalbumin	27	Cytochrome b_5	4.5
Epidermal growth factor	26	Insulin	4.4
Somatotropin	25	Calcitonin	4.3
Pancreatic ribonuclease	21	Neurophysin 2	3.6
Lipotropin beta	21	Plastocyanin	3.5
Haptoglobin alpha chain	20	Lactate dehydrogenase	3.4
Serum albumin	19	Adenylate cyclase	3.2
Phospholipase A_2	19	Triosephosphate isomerase	2.8
Protease inhibitor PST1 type	18	Vasoactive intestinal peptide	2.6
Prolactin	17	Corticotropin	2.5
Pancreatic hormone	17	Glyceraldehyde 3-P DH	2.2
Carbonic anhydrase C	16	Cytochrome C	2.2
Lutropin alpha chain	16	Plant ferredoxin	1.9
Hemoglobin alpha chain	12	Collagen	1.7
Hemoglobin beta chain	12	Troponin C, skeletal muscle	1.5
Lipid-binding protein A-II	10	Alpha crystallin B-chain	1.5
Gastrin	9.8	Glucagon	1.2
Animal lysozyme	9.8	Glutamate DH	0.9
Myoglobin	8.9	Histone H2B	0.9
Amyloid A	8.7	Histone H2A	0.5
Nerve growth factor	8.5	Histone H3	0.14
Acid proteases	8.4	Ubiquitin	0.1
Myelin basic protein	7.4	Histone H4	0.1

[a]percent/100 My
From (Nei, 1987; Dayhoff *et al.*, 1978)

pared to the loss of information that occurs when one compares DNA sequences. If the biological sequence of interest encodes a protein, protein sequence comparison is always the method of choice.

Figure 10: The limits of sequence similarity

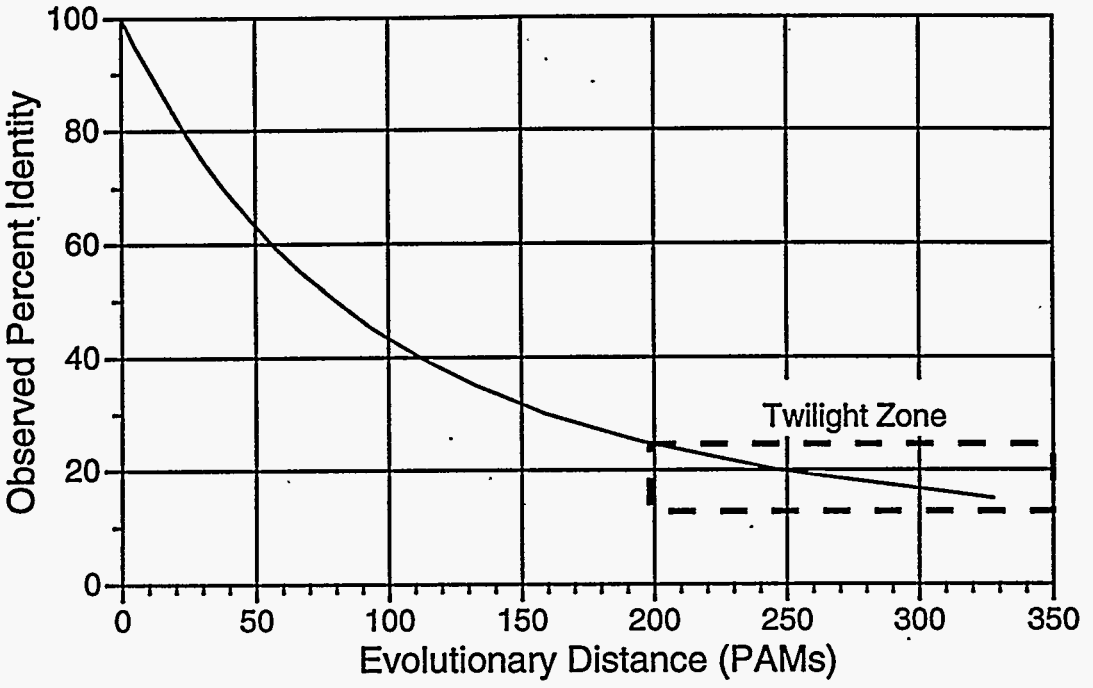


Table 5: Classification of Protein Families

I. Ancient Proteins

A. First editions. Direct-line descendacy to human and contemporary prokaryotes. Mostly mainstream metabolism enzymes. Example: triosphosphate isomerase (46%) identical.

B. Second edition. Homologous sequences in human and prokaryotic proteins, but apparently different functions. Example: human glutathione reductase and pseudomonas mercury reductase (27% identical).

II. Middle-age proteins. Proteins found in most eurkaryotes but prokaryotic counterparts are unknown. Example: actin.

III. Modern proteins

A. Recent vintage. Proteins found in animals or plants but not both. Not found in prokaryotes. Example: collagen.

B. Very recent inventions. Proteins found in vertebrates but not elsewhere. Example: plasma albumin.

C. Recent mosaics. Modern proteins clearly the result of exon shuffling. Example: LDL receptor.

From Doolittle *et al.*, 1986.

Table 6: Ancient human proteins

A. First edition type		
Human protein	Prokaryotic homologue	% identity
Triosephosphate isomerase	*E. coli*	46
Phosphoglyceraldehylde dehydrogenase	*B. stearothermophilus*	52
Alkaline phosphatase	*E. coli*	31
Dihydrofolate reductase	*E. coli*	30
Superoxide dismutase (Cu-Zn)	*P. leiognathi*	26
B. Second edition type		
Glutathione reductase	Mercuric reductase, *Pseudomonas*	27
Glutamate dehydrogenase (NAD)	Glutamate dehydrogenase,*E. coli*	26
Ornithine transcarbamylase	Aspartate ranscarbamylase, *E. coli*	26
Hypoxanthine-guanine phosphoribosyl transferase	Glutamine phosphoribosyl-PP_i transferase, *E. coli*	19

From Doolittle *et al.*, 1986

Table 7: Mosaic proteins

A. EGF-type	B. C9-type
Epidermal growth factor precursor	Complement C9
Tumor growth factors	LDL receptor
LDL receptor	Notch (*Drosophila*)
Factor IX	*lin-12* (*C. elegans*)
Protein C	
Tissue plasminogen activator	C. Fibronectin finger
Urokinase	Fibronectin
Complement C9	Tissue plasminogen activator
Notch protein (*Drosophila*)	
lin-12 (*C. elegans*)	D. Protease "Kringle"
	Plasminogen
	Tissue plasminogen activator
	Urokinase
	Prothrombin

From Doolittle *et al.*, 1986.

Figure 11: Structures of mosaic proteins

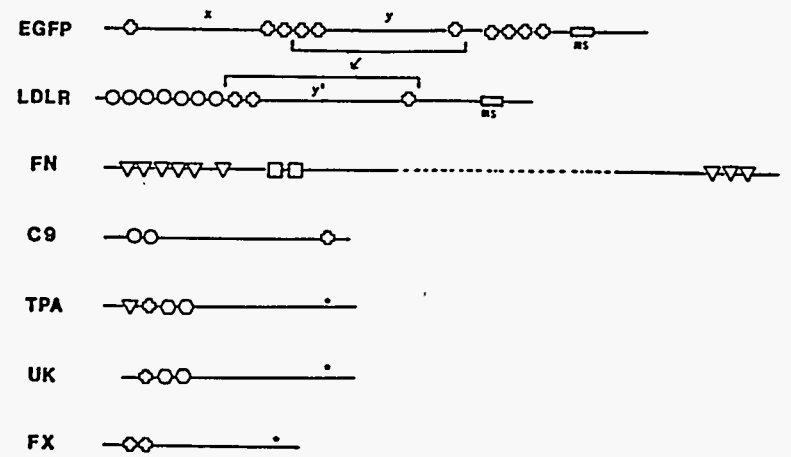


Figure 2. Some modern "mosaic" proteins that provide examples of exon shuffling. (EGFP) Epidermal growth factor precursor; (LDLR) low-density lipoprotein receptor; (FN) fibronectin; (C9) complement component C9; (TPA) tissue plasminogen activator; (UK) urokinase; (FX) blood coagulation Factor X; (MS) membrane spanning units; (•) active site of serine proteases. The sections labeled x, y, and y' have homologous sequences, over and beyond the five modular units described in Fig. 3. (Reprinted, with permission, from Doolittle 1985.)

From Doolittle *et al.*, 1986.

Figure 12: Intron/Exon Boundaries and Structural Features

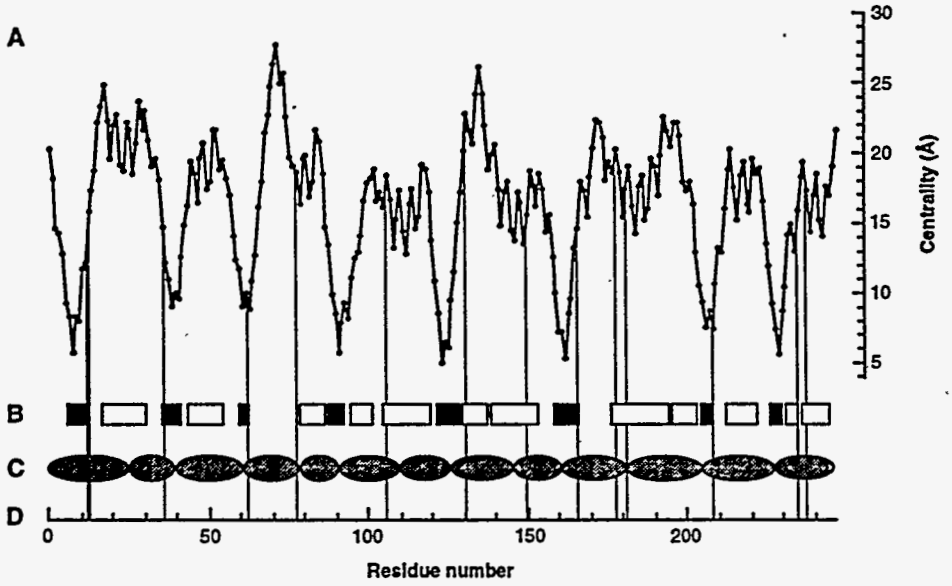


Fig. 4. Intron positions of TPI genes in relation to structural features of the 247-residue chicken muscle enzyme (*28*). **(A)** The centrality plot (average centrality, 16.2 Å) reveals the regularity of the β-barrel domain; the eight troughs represent the eight β strands that pass near the center, whereas the zigzagging segments between the troughs show the course of the peptide backbone as it winds through the peripheral α helices [see also domain A of PK (Fig. 3)]. **(B)** Elements of secondary structure. **(C)** Modules proposed by Gō and Nosaka (*15*). The 14 known intron positions **(D)** are represented in four genomic sequences (*27*) as follows: chicken, 37–1, 78–2, 107–0, 151–1, 180–0, 209–1; maize, 14–0, 37–1, 78–2, 107–0, 151–1, 183–0, 209–1, 237–0; *Aspergillus*, 13–2, 107–0, 132–0, 167–2, 239–1; mosquito, 64–0. Other conventions are as indicated for Fig. 1.

From Stoltzfus *et al.*, 1994.

Table 8: DNA vs Protein Sequence Comparison

		score	E(DNA)	E(prot)
MUSGLUTA	Mouse glutathione S-transferase class mu	5625	0	0
MUSGSTA	Mouse, glutathione transferase GT9.3 mu	3953	0	0
HUMGSTAA	Homo sapiens glutathione transferase	1257	0	0
MAMGLUTRA	M.auratus mu class GST	399	10^{-11}	0
RATGSTYD	Rat glutathione S-transferase Yb subunit	399	10^{-11}	0
HSGSTM4	H.sapiens GSTM4 gene for GST	390	10^{-10}	0
RATGSTY	Rattus norvegicus GST	372	10^{-9}	0
HSGSTM1B	H.sapiens GSTM1b gene for GST	358	10^{-9}	0
HSGSTMU3	Human GSTmu3 gene for a GST	322	10^{-7}	
HSGST145	Human GST-1 gene for GST	308	10^{-6}	
BTGST	Bovine GST mRNA for GST	249	0.0002	10^{-16}
HSGSTPI1	Human mRNA for anionic GST	237	0.0008	10^{-17}
MUSGTF	Mus musculus GST mu	196	0.06	
CRUGSTP	Chinese hamster GST	196	0.06	10^{-16}
CRUGSTPIE	Cricetulus griseus GST pi	196	0.06	10^{-16}
HAMGSTPIE	Mesocricetus auratus GST pi	191	0.1	10^{-16}
RRGTS8	R.rattus mRNA for GST	182	0.2	
HUMKAL2	*Human glandular kallikrein gene*	*170*	*0.6*	
HUMTROPI01	*Human troponin I, slow-twitch isoform*	*170*	*0.8*	
RNGSTYC2F	R.norvegicus GST Yc2	170	0.8	10^{-7}
MMGLUT	M.musculus mRNA for GST	168	1.1	10^{-7}
MUSTHYGP	*Mouse Thy-1.2 glycoprotein*	*163*	*1.3*	
HUMLGTH1	Human liver glutathione S-transferase	157	3.4	10^{-5}
ATCON430S1	*Rattus norvegicus connexin*	*155*	*3.6*	
HUMA1AR2	*Human a-1-antitrypsin-related protein*	*154*	*3.6*	
HUMVLDLR	*Human VLDL protein receptor*	*152*	*4.5*	
RABGSTB	Oryctolagus cuniculus glutathione S-tr	153	5.1	10^{-9}
HUMHSF1	*Human heat shock factor 1 (TCF5)*	*151*	*5.5*	
RATRIIA	*Rat type I reg. subunit of cAMP*	*151*	*5.9*	
RNGSTYC1F	R.norvegicus GST Yc1	148	8.5	10^{-6}
RATGSTYC	Rat liver glutathione S-transferase Yc	148	8.6	10^{-6}
MUSCX43GA	*Mouse Cx43 gene, exon 1.*	*147*	*11*	
HUMTAN1	*Human TAN-1 mRNA (homologue of Drosoph*	*142*	*12*	
OCDHPR	*Rabbit mRNA for dihydropyridine (DHP)*	*142*	*12*	
A01444	*Human DNA for 4.6 kb retinoblastoma*	*142*	*12*	
HUMGSTB	Human glutathione S-transferase	144	14	
HUMGSTH	Human glutathione S-transferase	144	14	10^{-6}
HUMGST2	Human glutathione S-transferase 2	144	14	10^{-6}
S49975	Human glutathione transferase A1-1	144	14	10^{-6}

Expectation values for searches against DNA (score, E(DNA)) and protein databases. A mouse glutathione transferase cDNA sequence (MUSGLUTA) was used to search either the primate (GBPRI), rodent (GBROD), and mammalian (GBMAM) divisions of the GenBank DNA sequence database for the DNA sequence comparisons. Protein expectations (E(prot)) were calculated from a search the translated cDNA sequence against the GenPept sequence database, which includes all of translated GenBank. Unrelated sequences are *italicized*; E(prot) for unrelated sequences are >> 100.

2 Alignment methods

A variety of comparison algorithms and scoring parameters can be used to evaluate protein or DNA sequence similarity. In general, the choice the of "best" algorithm depends on the problem to be solved. Thus, algorithms that calculate a local comparison score—i.e., they find the strongest similarity between the two sequences, ignoring differences outside of the most similar region—are usually most appropriate for searching protein and DNA databases,[2] while global comparison algorithms are more appropriate when homology has been established, as when building evolutionary trees. Pattern-based, rather than similarity-based, comparison methods may be preferred when searching for functionally conserved non-homologous domains.

In searching protein sequence databases to identify distantly related homologous proteins, it is important to remember that avoiding high similarity scores with unrelated sequences can be more important as calculating high scores for related sequences. There are more than 40,000 protein sequences in comprehensive protein databases, while the typical family of proteins has fewer than 100 members. Thus, comparison algorithms, scoring matrices and gap penalties that produce the best alignments may not be the most effective for searching protein sequence databases (Pearson, 1995).

2.1 Algorithms

Two general classes of comparison algorithms are used to calculate similarity scores to infer sequence homology: rigorous algorithms that are guaranteed to calculate an optimal similarity score, e.g. the NeedlemanWunsch (Needleman & Wunsch, 1970) and SmithWaterman (Smith & Waterman, 1981) algorithms, and rapid heuristic algorithms that do not guarantee to calculate an optimal score for every sequence in a library, e.g. FASTA (Pearson & Lipman, 1988) and BLAST(Altschul *et al.*, 1990). Table 2.1 summarizes widely used algorithms for biological sequence comparison.

Two optimal algorithms for calculating similarity scores have been described, the NeedlemanWunsch algorithm (Needleman & Wunsch, 1970), which calculates a "global" similarity score between two sequences, and the Smith-Waterman algorithm (Smith & Waterman, 1981), which calculates a "local" similarity score. Global scores require the alignment to begin at the beginning of each sequence and extend to the end of each sequence. Global alignments cannot be used to detect the relationship between DNA binding domains in homeobox proteins or the calcium binding domains shared between calmodulin and calpain. Likewise, global alignment algorithms often do not detect the relationships between mosaic proteins. Global similarity scores can be calculated with or without penalties for gaps at the ends of the sequences.

Local alignment algorithms identify the most similar region shared between two sequences. Thus, homologous calcium binding domains embedded in non-homologous proteins can be detected with local alignment methods. In addition, a local alignment algorithm can be used

[2]For genomic DNA sequences, there is no logical alternative.

Table 9: Algorithms for comparing protein and DNA sequences .

algorithm	value calculated	scoring matrix	gap penalty	time required	
Needleman-Wunsch	global similarity	arbitrary	penalty/gap q	$O(n^2)$	Needleman and Wunsch, 1970
Sellers	(global) distance	unity	penalty/residue rk	$O(n^2)$	Sellers, 1974
Smith-Waterman	local similarity	$\hat{S}_{ij} < 0.0$	affine $q + rk$	$O(n^2)$	Smith and Waterman, 1981 Gotoh, 1982
FASTA	approx. local similarity	$\hat{S}_{ij} < 0.0$	limited gap size $q + rk$	$O(n^2)/K$	Lipman and Pearson, 1985 Pearson and Lipman, 1988
BLASTP	maximum segment score	$\hat{S}_{ij} < 0.0$	multiple segments	$O(n^2)/K$	Altshul et al., 1990

to find the exons in a genomic DNA sequence by aligning it with its encoded mRNA. Local alignment algorithms are required to identify homologies in mosaic proteins, and they can be used to detect internal domain duplications as well. Table 10 compares the scores of global, global without end-gap-penalties, and local similarity scores for a variety of related and unrelated proteins.

Rigorous sequence comparison algorithms, like the Smith-Waterman algorithm, require time proportional to $O(mN)$, where m is the length of the query sequence and N is the number of amino acids in the protein sequence library. Modern high-performance unix workstations can compare a 300 residue protein sequence (human opsin) to the 40,000 entry, 15,000,000 amino acid Swiss-Prot 31 database in less than 10 minutes.

Although very rapid[3] algorithms are available for calculating optimal global similarity scores between two sequences, particularly with unit cost scores, such algorithms are rarely appropriate for biological sequence comparison. Unit cost algorithms must discard the substantial biochemical information encoded in the PAM250 matrix. Most rapid optimal algorithms calculate only global similarities; such comparisons are not useful for DNA sequence comparison because the "ends" required for a global sequence comparison are usually arbitrary.

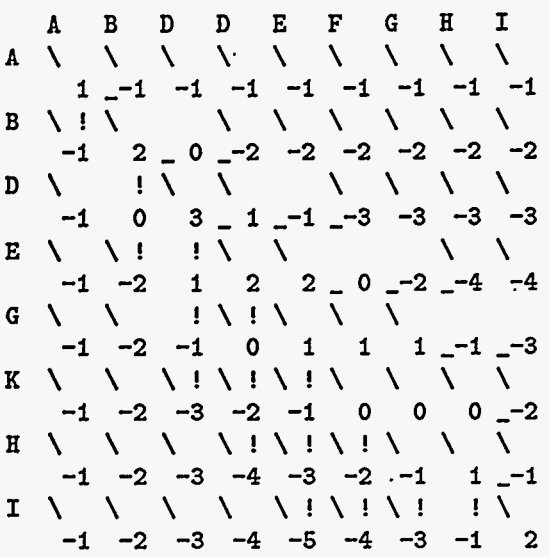
[2] $O(Nd)$, where N is the length of a sequence and d is the number of differences between the two sequences.

Table 10: Global and local sequence similarity scores

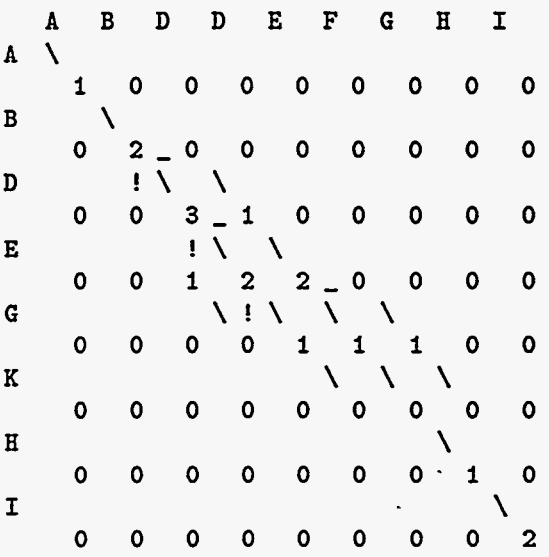
PIR Entry				Similarity Score			Distance
				Global		Local	
				End Penalty	No End Penalty		
HBHU	vs	HBHU	Hemoglobin beta-chain—human	725	725	725	0
		HAHU	Hemoglobin alpha-chain—human	314	320	322	152
		MYHU	Myoglobin—Human	121	164	166	212
		GPYL	Leghemoglobin—Yellow lupin	8	28	43	239
		LZCH	Lysozyme precursor—Chicken	−107	16	32	220
		NRBO	Pancreatic ribonuclease—Bovine	−124	16	31	280
		CCHU	Cytochrome c—Human	−160	10	26	321
MCHU	vs	MCHU	Calmodulin—Human	671	671	671	0
		TPHUCS	Troponin C, skeletal muscle	395	430	438	161
		PVPK2	Parvalbumin beta—Pike	−57	103	115	313
		CIHUH	Calpain heavy chain—Human	−2085	89	100	2463
		AQJFNV	Aequorin precursor—Jelly fish	−65	48	76	391
		KLSWM	Calcium binding protein—Scallop	−89	45	52	323
QRHULD	vs	EGMSMG	Epidermal growth factor precursor	−591	475	655	2549

Figure 13: Global and local alignment paths

A. Global



B. Local



Optimal global alignments (score 2):

```
    A B D D E G K H I  (top)
    A B D - E G K H I  (side)
or  A B - D E G K H I
```

Optimal local alignment (score 3):

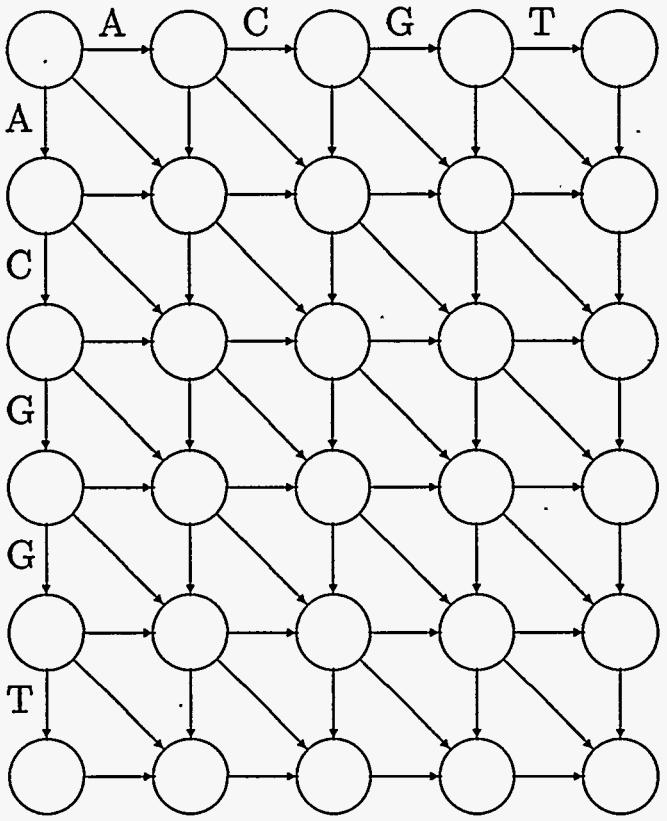
```
  A B D  (top)
  A B D  (side)
```

2.2 Dynamic Programming Algorithms

The algorithms used to calculate the maximum similarity scores between two sequences are most easily visualized with an alignment matrix or path graph. Figs. 13–14 demonstrate the correspondence between an alignment path graph and an actual alignment. The goal along the path is to maximize the similarity score for the alignment that ends at each potential vertex. For the figures, similarity scores are increased by +1 for diagonal edges if the the two residues along the path are identical; the the are different, the diagonal edge cost is −1. The cost along either a vertical or horizontal edge, which corresponds to an insertion in the top sequence (vertical edge) or an insertion in the left-side sequence (horizontal edge) is −2. To produce a global alignment from a path graph, simply begin at the bottom-right corner of the graph and follow the "active" paths, noted by \, _ or ! to the upper-left corner, aligning the two residues along the diagonal path, or aligning a residue with a gap if a horizontal or vertical path is taken.

For the global alignment in Fig. 13A, there are two alignments that produce the optimal score. Optimal comparison algorithms guarantee to produce the best score, given the match, mismatch, and gap costs, but frequently there are several optimal alignments for a single score. For the local alignment in Fig. 13B, there are several sub-optimal alignments with

Figure 14: An alignment path matrix



scores of 2. Note that the local alignment in Fig. 13B would extend from one end of each sequence to the other if the gap cost were reduced to -1.

Fig. 14 provides an exercise for the reader.

While there are an exponential number of potential alignments with gaps between two protein or DNA sequences, dynamic programming algorithms are available that can calculate the optimal score in $O(MN)$ steps. This efficiency is achieved by determining the optimal score for each prefix of each string, and then extending each prefix by considering the three paths that can be used to extend an alignment: (1) by extending the alignment by one residue in each sequence; (2) by extending the alignment by one residue in the first sequence and aligning it with a gap in the second; or (3) extending the alignment by one residue in the second sequence and aligning it with a gap in the first. This decision must be made for each of the MN prefixes of sequences of length M and N.

The first algorithm for comparing protein sequences (Needleman & Wunsch, 1970) calculates a "global" similarity score. A simplified global algorithm is shown in Fig. 15. Since a global algorithm requires that the alignment extend from the beginning to the end of the

Figure 15: Algorithms for Global and Local similarity scores

$S(0,0) \leftarrow 0$
for $j \leftarrow 1$ *to* N **do**
    $S(0,j) \leftarrow S(0,j-1) + \sigma(\ \overline{\ }\ _{b_j})$
for $i \leftarrow 1$ **to** M **do**
[$S(i,0) \leftarrow S(i-1,0) + \sigma(\ a_i\ _{\ \overline{\ }})$
    for $j \leftarrow 1$ **to** N **do**
        $S(i,j) \leftarrow max[S(i-1,j-1) + \sigma(\ a_i\ _{b_j}), S(i-1,j) + \sigma(\ a_i\ _{\ \overline{\ }}), S(i,j-1) + \sigma(\ \overline{\ }\ _{b_j})]$
]
write "Global similarity score is" $S(M,N)$

$best \leftarrow 0$
for $j \leftarrow 1$ **to** N **do**
    $S'(0,j) \leftarrow S'(0,j-1) + \sigma(\ \overline{\ }\ _{b_j})$
for $i \leftarrow 1$ **to** M **do**
[$S'(i,0) \leftarrow S'(i-1,0) + \sigma(\ a_i\ _{\ \overline{\ }})$
    for $j \leftarrow 1$ **to** N **do**
    [$S'(i,j) \leftarrow max[0, S'(i-1,j-1) + \sigma(\ a_i\ _{b_j}), S'(i-1,j) + \sigma(\ a_i\ _{\ \overline{\ }}), S'(i,j-1) + \sigma(\ \overline{\ }\ _{b_j})]$
        $best \leftarrow max(S'(i,j), best)$
]
write "Local similarity score is" *best*

alignment, it is sufficient to report the score in the lower right ($S(M,N)$) of the scoring matrix.

Local alignment algorithms must consider alignments that begin and end at each of the MN positions in the alignment matrix. Despite this added complexity, they only add two additional steps to the global alignment algorithm. Since every possible starting position must be considered, similarity scores cannot fall below zero and a 0 term is added to the *max* comparison in Fig. 15. Since they can end at any position in the matrix, the *best* score must be saved at each step. In practice, global and local comparison algorithms require the same amount of computation.

2.3 Scoring methods

The scoring matrices used for protein sequence comparison are much more sophisticated than +1 for a match and −1 for a mismatch. The most effective matrices are based on the actual frequency of substitutions that occur between related proteins. Two different approaches have been used to produce these matrices. The original PAM250 matrix (Fig. 8) was produced by examining several hundred alignments between very closely related proteins, and then calculating the frequency with which each amino-acid residue changed into each of the others at a very short evolutionary distance—one where only 1% of the residues had kchanged (Dayhoff *et al.*, 1978). This replacement frequency, when corrected for the amino-acid abundance, can be used to calculate the PAM1 scoring matrix (PAM is "Point Accepted Mutation"). If the matrix is multiplied against itself 250 times, a PAM250 matrix, which reflects the frequency of change for proteins that have diverged 250%. If a two protein sequences have diverged by 250%, it is expected that they will share about 20% sequence identity (Fig. 10). Since 20% identity is at the edge of where significant similarity can be detected, the PAM250 matrix has been widely used. The PAM250 matrix is based on small number of amino acid substitutions; modern extrapolated matrices based both on sequence alignments (Jones *et al.*, 1992) and structural alignments (Johnson & Overington, 1993) are available.

Substitution matrices have also been calculated directly by examining "blocks" of aligned sequences that differ by no more than X% (Henikoff & Henikoff, 1992). Thus, the BLOSUM62 matrix, which is used by the BLASTP rapid comparison program, is derived from substitution data for blocks of aligned sequences that are no more than 62% identical. BLOSUM62 performs substantially better than extrapolated matrices with BLASTP and FASTA (Henikoff & Henikoff, 1993), but both BLOSUM and extrapolated matrices can perform well when used with optimal gap penalties (Pearson, 1995).

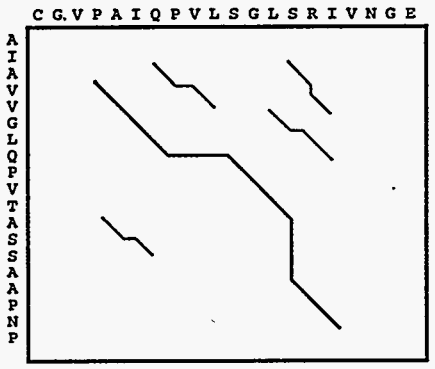
Altschul (1991) has provided a information-theory based perspective for evaluating scoring matrices in general for alignments without gaps. Using a statistical theory for such alignments (Karlin & Altschul, 1990), it is possible to convert any similarity score to a value in "bits" that can be used to compare scores produced by different alignments. Unfortunately, the analytical formulas that are used for this conversion cannot easily be applied to alignments that contain gaps. Collins *et al.*, 1988 and Altschul, 1993 have also pointed out that different scoring matrices are optimal at different evolutionary disances. Thus, short proteins sequences that are 50% identical can be more easily identified with a "shallower" PAM matrix, e.g. PAM60.

2.4 Heuristic Algorithms

Two rapid heuristic algorithms are frequently used for searching protein and DNA sequence databases, FASTA (Pearson & Lipman, 1988) and BLASTP (Altschul *et al.*, 1990). These methods are 5–50 times faster than the rigorous Smith-Waterman algorithm, and can produce results of similar quality in many cases.

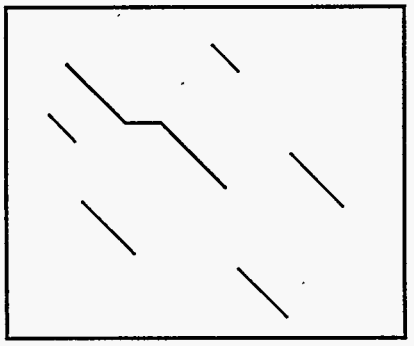
Fig. 16 summarizes the difference between the FASTA, BLASTP, and Smith-Waterman algorithms. BLASTP and FASTA are faster than Smith-Waterman because they examine

Figure 16: Heuristic strategies for sequence comparison



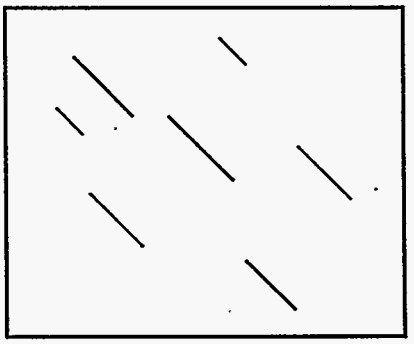
Smith-Waterman

time: 10:00 min



FASTA

time: 2:00 min



BLAST

time: 20 sec

Table 11: Sequence similarity with BLASTP

Step 1 For each three amino acids in the query sequence, identify all of the substitutions of each word that have a similarity score greater than a threshold score $T = 11$. In practice, word-matches with scores $\geq T$ are seen on average 150 times per library sequence.

Step 2 Build a discrete finite automaton (DFA) to recognize the list of identical and substituted three letter words.

Step 3 Use the DFA to identify all of the matching words in sequences in the database. If a match is found, attempt to extend the match both forwards and backwards using the BLOSUM62 matrix to produce a score that is higher than a threshold score. Save all of the high scoring regions shared by the query sequence and each library sequence. The best of these scores is reported as the best single MSP (maximal segment pair) score. These high scoring regions do not contain gaps.

Step 4 Attempt to combine multiple MSP regions. For each "consistent" combination, calculate the probability of obtaining that may consistent matches using either "poisson" or "sum" statistics.(Karlin & Altschul, 1993) Report the lowest probability score based on statistics used.

Step 5 Report all of the significant alignments. Frequently, a query and library sequence will contain several MSPs because of the requirement that they do not contain gaps.

only a portion of the potential alignments between two sequences. FASTA focuses on regions where there are either pairs (*ktup=2*) or single aligned *ktup=1* identities; BLASTP examines regions that include triples of conserved amino acids.

2.4.1 BLAST

Advances in the statistical theory of sequence alignments without gaps (Karlin & Altschul, 1990) provided the theoretical basis for the BLASTP program (Altschul *et al.*, 1990). BLASTP is now the most widely used program for rapid sequence comparison, in large part because of its accurate estimates for the statistical significance of similarity scores (see 3. BLASTP, like FASTA, uses a word-based scanning procedure to identify regions of local similarity (11) with out gaps. BLASTP is effective because it combines high sensitivity with excellent selectivity. BLASTP combines good sensitivity with exceptional selectivity. Except when the query sequence contains a low complexity region, BLASTP rarely calculates scores for unrelated sequences.

2.4.2 FASTA

The current version of FASTA provides several significant improvements over earlier versions. FASTA now calculates optimized scores (step 4 in Table 12)) for most of the sequences in the database and provides accurate estimates for statistical significance (3). Calculation of optimized scores improves substantially the performance of FASTA. Without the calcula-

Table 12: Sequence similarity with FASTAv20

Step 1 Identify regions shared by the two sequences with the highest density of identities (*ktup=1*) or pairs of identities (*ktup=2*).

Step 2 Rescan the ten regions with the highest density of identities using the BLOSUM50 matrix. Trim the ends of the region to include only those residues contributing to the highest score. Each region is a partial alignment without gaps.

Step 3 If there are several initial regions with scores greater than the CUTOFF value, check to see whether the trimmed initial regions can be joined to form an approximate alignment with gaps. Calculate a similarity score that is the sum of the joined initial regions minus a penalty (usually 20) for each gap (*initn*). The score of the single best initial region found in Step 2 is also reported (*init1*).

Step 4 For sequences with scores greater than a threshold, construct an optimal local alignment of the query sequence and the library sequence, considering only those residues that lie in a band centered on the best initial region found in Step 2. For protein searches with *ktup=2* a 16 residue band is used by default. A 32 residue band is used with *ktup=1*. This is the optimized (*opt*) score.

Step 5 After all (or the first 10–20,000) scores have been calculated, normalize the raw similarity scores by regressing the similarity score against ln(library-sequence length) and calculating the average variance in similarity scores. *Z-values* (normalized scores with mean 0 and variance 1) are calculated, and the calculation is repeated with library sequences with *z-values* greater than 5.0 and less than -5.0 removed. These z-values are used to rank the library sequences.

Step 6 The Smith-Waterman algorithm (without limitation on gap size) is used to display alignments.

tion, FASTA performs significantly worse than BLASTP; however, with the calculation of optimized scores (and normalization of the scores based on library sequence length), FASTA performs significantly better than BLASTP and almost as well as the Smith-Waterman algorithm (Pearson, 1995). In addition, FASTA now uses the Smith-Waterman algorithm to produce final alignments; previous versions limited the size of gaps, which sometimes led to incomplete alignments.

Every database search for members of a diverse protein family involve a tradeoff between sensitivity—the ability to identify distantly related members of the family—and selectivity—the ability to avoid high similarity scores for unrelated sequences. Table 3.3 compares how effectively the three algorithms maintain this balance for a large protein family—the G-protein-coupled receptors. Thus, BLASTP calculates a very highly significant score for the closely related opsin and dopamine D2 receptors, and a significant score for the more distantly related thromboxane A_2 receptor, but it does not detect the similarity between opsin and the very distantly related *Dictyostelium* cAMP (CAR1) receptor. In addition, BLASTP would never suggest a relationship between opsin and cytochrome oxidase. FASTA (*ktup=2* does a better job at recognizing the relationship between opsin and thromboxane A2, fails to detect the cAMP-1 receptor, and is more ambiguous about a possible relationship with cytochrome oxidase. FASTA with *ktup=1* and Smith-Waterman calculate statistically signif-

icant relationships between opsin and cAMP-1, but also good (but not significant) scores for opsin and cytochrome oxidase.

3 The statistics of sequence similarity scores

The development of accurate statistical estimates for local sequence similarity scores (Karlin & Altschul, 1990; Mott, 1992) has allowed dramatic improvement in our ability to reliably recognize distantly related proteins. The statistical estimates calculated by BLASTP are used widely in large scale sequence comparison, e.g. to characterize all of the genes on a yeast chromosome or all of the genes in a bacterial genome. The incorporation of statistical estimates into FASTA and SSEARCH (a Smith-Waterman implementation) have significantly improved the performance of these programs as well.

3.1 Sequence alignments without gaps

The statistics of local similarity scores for alignments without gaps but with an arbitrary substitution matrix have been described by Karlin & Altschul, 1990. Local similarity scores are described by the *extreme value* distribution. Using the parameters λ and K, which can be derived from the scoring matrix and the amino acid composition of the query sequence, the probability that a normalized similarity score:

$$S' = \lambda S - \ln Kmn \tag{1}$$

(Karlin & Altschul, 1990; Altschul *et al.*, 1994) where m is the length of the query sequence and n is the length of the library sequence can be calculated as:

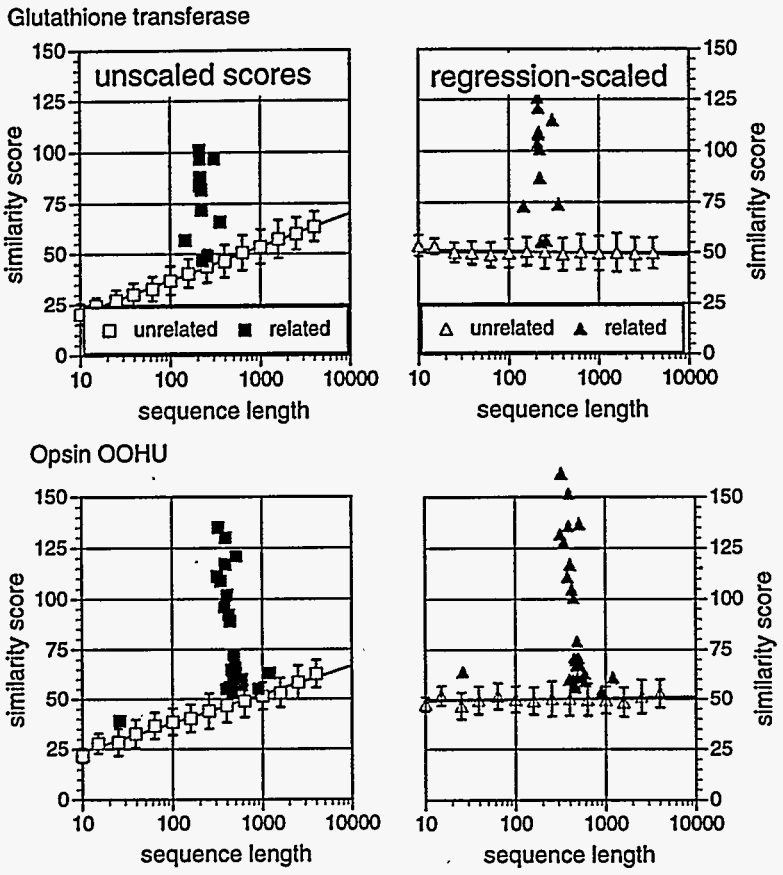
$$P(S' \geq x) = 1 - exp(-e^{-x}) \tag{2}$$

Since a typical database search typically involves thousands of pairwise comparisons, the expectation of finding a score $S' \geq X$ for a search of D sequences is: $E(S' \geq X) = PD$. (Thus, searches of highly redundant databases may be less informative, because D is larger but the number of different sequences is not.)

3.2 Similarity scores increase with sequence length

The normalization in equation 1 shows that scores for alignments without gaps between random sequences increase as $\ln Kmn$, or since K and m are fixed for a given search, $\ln n$, the length of the library sequence. This is seen empirically with scores for alignments that contain gaps (Collins *et al.*, 1988; Mott, 1992) and is shown in Fig. 17. For local similarities, the variance of the score should be independent of library sequence length. Thus, normalization of similarity scores by fitting a line to the relationship of similarity score to $\ln n$ will reduce the scores of long, unrelated sequences, and make it possible to detect more distant relationships (Pearson, 1995).

Figure 17: Similarity scores and library sequence length



The distribution of Smith-Waterman similarity scores is plotted as a function of $\log(n)$, n is the length of the library sequence. Filled symbols indicate individual related sequences (only the most distant related sequences are shown); open symbols show the average and std. error of similarity scores for unrelated sequences.

3.3 Empirical statistics for alignments with gaps

Accurate statistical estimates for alignments with gaps can can be calculated by normalizing similarity scores to remove the $\ln n$ dependence for similarity scores. This can be seen in Fig. 5, where the '*'s show the fit of an extreme value distribution to the observed data ('=='). FASTA and SSEARCH estimate statistical significance by fitting a line to S vs $\ln n$ and calculating the average variance for the scores. The regression line and variance are used to calculate

$$Z - score = (S - (a + b \ln n))/var \tag{3}$$

The distribution of $Z - score$'s should follow the extreme value distribution, so that:

$$P(Z > x) = 1 - exp(-e^{-1.282Z - 0.5772}) \tag{4}$$

and, as before, $E(Z > x) = PD$.

Table 13: Search Algorithms and Statistical Significance

algorithm	closely related	related	distantly related	unrelated
	dopamine D2[a]	thromboxane A2[b]	cAMP-1[c]	cytochrome oxidase[d]
Smith-Waterman	3×10^{-9}	2×10^{-4}	0.01	0.57
PRSS[e]	8×10^{-10}	10^{-4}	0.007	0.45
PRSS(window=20)[e]	8×10^{-8}	0.001	0.23	3.0
fasta, *ktup=1, opt*	3×10^{-9}	7×10^{-5}	0.02	0.39
fasta, *ktup=2, opt*	2×10^{-6}	10^{-4}	2.2	0.36
BLASTP	2×10^{-22}	0.07	> 1.0	> 1.0

[a]D2DR_HUMAN, [b]TA2R_MOUSE, [c]CAR1_DICDI, [d]APPC_ECOLI

Expected number of times that a similarity score as high or higher than that obtained by the indicated library sequence would be obtained by chance in a search of Swiss-Prot ($\approx 43,000$ entries) with the OPSD_HUMAN (human opsin) query sequence. [e]Expected times this score would be obtained after 1,000 shuffles of the indicated library sequence with either global (prss) or local (window=20) amino acid exchanges.

3.4 Statistical significance by random shuffling

Statistical estimates derived from database searches measure the difference between an observed similarity score and that expected for a sequence with the amino acid composition of the database. Such tests may overestimate significance in cases where the query sequence's amino acid composition differs from that of the database. Thus, membrane proteins with their hydrophobic transmembrane domains may have statistically significant scores with non-homologous membrane proteins. A more challenging test compares the similarity score between a query and library sequence with the distribution of scores obtained by comparing the query sequence to random sequences with the same length and amino acid composition as the library sequence. Such sequences are easily generated by randomly shuffling the library sequence, either globally, by exchanging randomly each amino acid with any other position in the sequence, or locally, by performing the exchanges within a window of 10–20 residues. Because this Monte Carlo test measures the significance of the order of the two amino acid sequences, rather than the difference between the highest scoring sequences and the rest of the database, it tends to be more demanding.

As before, similarity scores for random sequences should follow the extreme value distribution, and a fit of the distribution of scores can be used to estimate the significance of an unshuffled score. However, to extrapolate an expectation value from shuffled sequences to that for a library search, the "E()-value" must be multiplied by the ratio of the number of

sequences in the library to the number of shuffled sequences. Thus, in the example below, an E()-value from 500 shuffles must be multiplied by 80 to be comparable to an E()-value from the 40,000 entry Swiss-Prot. As expected, the E()-value from the actual search—2×10^{-4}—is slightly more significant than that from the shuffled distribution—3×10^{-3}.

```
 Comparison of OOHU (human opsin) with TA2R_MOUSE (thromboxane A2 receptor)
 BLOSUM50 matrix, gap penalties: -12,-2
 unshuffled s-w score: 160; shuffled score range: 38 - 92
Lambda: 0.15076 K: 0.017357; P(160)= 7.4282e-08
For 500 sequences, a score >=160 is expected 3.71e-05 times
```

Although accurate statistical estimates can be very valuable in interpreting the results of similarity searches, they must be evaluated with caution. Distantly related homologous sequences often do not share statistically significant similarity. Thus, over reliance on statistical estimates, particularly after a single search, can miss genuine homologies. Conversely, sequences with low-complexity regions often share significant similarity but are not homologous. Finally, some structures, such as the coiled-coil structure in tropomyosin, share statistical significance because of a common repeated structure, because of convergence (analogy), rather than homology.

4 Identifying distantly related protein sequences

In this section, we will examine similarity searches in three diverse families of protein sequences, serine proteases, glutathione S-transferases, and the G-protein-coupled receptors. The serine proteases are considered because they provide a classic example of a family of proteins with a highly conserved active site; the glutathione transferases are a very diverse family where many members do not share significant similarity with all other members, while the G-protein-coupled receptors are a very large and diverse family of membrane proteins.

4.1 Serine proteases

Serine proteases cleave peptide bonds using a "catalytic triad" of histidine, serine, and aspartic acid; these residues are underlined in Fig. 20. Because these residues are so highly conserved, patterns that focus on two of the regions (Fig. 18) can be used to identify every member of the serine protease family. Fig. 19 shows the highest scoring unnormalized similarity scores. As is often the case for divergent protein families, several members of the family do not share statistically significant similarity with bovine trypsin. These sequences are italicized in Fig. 19; their membership in the serine protease family is based on common three-dimensional structures. As expected from the discussion in section 3.2, several of the highest scoring unrelated sequences are substantially longer than genuine serine proteases. These scores have much higher (less significant) expectation values when the $\ln n$ correction is used.

Figure 18: Patterns for serine proteases

```
ID   TRYPSIN_HIS; PATTERN.
AC   PS00134;
DE   Serine proteases, trypsin family, histidine active site.
PA   [LIVM]-[ST]-A-[STAG]-H-C.
NR   /TOTAL=158(158); /POSITIVE=154(154); /UNKNOWN=2(2); /FALSE_POS=2(2);
NR   /FALSE_NEG=11(11);
CC   /TAXO-RANGE=??EP?; /MAX-REPEAT=1;
CC   /SITE=5,active_site;

ID   TRYPSIN_SER; PATTERN.
AC   PS00135;
DE   Serine proteases, trypsin family, serine active site.
PA   G-D-S-G-G.
NR   /TOTAL=160(160); /POSITIVE=151(151); /UNKNOWN=1(1); /FALSE_POS=8(8);
NR   /FALSE_NEG=16(16);
CC   /TAXO-RANGE=??EP?; /MAX-REPEAT=1;
CC   /SITE=3,active_site;
```

Patterns from PROSITE that identify 152/163 (TRYPSIN_HIS or 143/159 TRYPSIN_SER members of the serine protease protein family.

The absolute conservation of residues in the "catalytic triad" might suggest that similarities between members of this family are limited to those regions. This is not the case, as can be seen in Figs. 20. Similarity in the serine proteases typically extends from one end of the protein to the other, with strong conservation throughout the sequence. Indeed, the region around one of the residues in the catalytic triad—the apartic acid—is not well conserved. While the residues in the catalytic triad is an essential feature of serine proteases, the serine protease fold (two domains containing anti-parallel β-barrels) are required to bring these residues together.

The requirement for a common folded structure in homologous proteins usually causes similarities to extend from one end of the protein to the other, or for mosaic proteins, from one end of a domain to the other. Fig. 21 displays the locally similar regions for the related and unrelated in Table 19; the highest scoring unrelated sequences tend to have relatively short (< 100 residue) regions of higher similarity ($\approx 30\%$ identical) while related sequences have longer (140–400), though sometimes lower (25%) similarity. In general, shorter, higher similarities are less significant than longer, lower similarities.

Figure 19: Serine protease search - high scoring sequences

LOCUS	Description	len	score	E(10,000)
TRBOTR	trypsin precursor - bovine	229	1559	0
TRRT2	trypsin II precursor - rat	246	1240	0
KQHU	tissue kallikrein precursor -	262	669	4.46×10^{-38}
NGMSG	7S NGF gamma chain I	237	645	1.46×10^{-36}
KQRTTN	tonin - rat	235	623	4.09×10^{-35}
KYBOA	chymotrypsin A precursor - bovine	245	609	3.66×10^{-34}
PLHU	plasmin precursor - human	790	580	1.71×10^{-31}
TRFF	trypsin-like proteinase	256	579	3.73×10^{-32}
KFHU	coagulation factor IXa	461	578	1.04×10^{-31}
ELRT2	pancreatic elastase II	271	559	8.46×10^{-31}
KYBOB	chymotrypsin B precursor - bovine	245	556	1.15×10^{-30}
KFHU1	coagulation factor XIa	625	547	1.77×10^{-29}
WMMS28	complement factor D homolog	259	541	1.22×10^{-29}
EXBO	coagulation factor Xa	492	518	1.01×10^{-27}
DBHU	complement factor D	246	517	4.33×10^{-28}
KXBO	protein C (activated)	456	515	1.42×10^{-27}
UKHU	u-plasminogen activator precu	431	507	4.41×10^{-27}
TBHU	thrombin precursor - human (fr	615	472	1.45×10^{-24}
TRSMG	trypsin - Streptomyces griseus	221	409	5.03×10^{-21}
C1HURB	complement subcomponent C1r p	705	356	7.14×10^{-17}
HPHU1	haptoglobin-1 precursor - human	347	335	6.9×10^{-16}
TRPGAZ	azurocidin - pig	219	316	6.9×10^{-15}
HPRT	haptoglobin - rat (fragments)	297	289	6.1×10^{-13}
C2HU	complement C2 - human	752	198	1.8×10^{-06}
BBHU	complement factor B - human	739	169	0.00014
KXBOZ	protein Z - bovine	396	142	0.0041
TRYXB4	alpha-lytic proteinase	396	107	0.83
OKBY8W	probable protein kinase YCR008W	603	107	1.3
RRIHM2	RNA-directed RNA polymerase	4488	99	37
IJFFTM	cadherin-related tumor suppressor	5147	99	42
GNNYE7	genome polyprot. - enterovirus 70	2194	98	20
VGIHHC	E2 glycoprotein - coronavirus	1173	96	14
QRRBVD	VLDL receptor - rabbit	873	96	10
PRSMBG*	proteinase B - S. griseus	185	96	1.9
MMMSB2	laminin chain B2 precursor - mouse	1607	95	23
RERTK	renin precursor - rat	402	94	6.0
MMMSA	laminin chain A - mouse	3084	93	61
LNRZ	lectin precursor - rice	227	90	6.0
PRSMAG*	proteinase A - S. griseus	182	89	5.5

Figure 20: Alignment of serine proteases

```
TRSMG trypsin (EC 3.4.21.4) precursor - Streptomyces griseus (259 aa)
Smith-Waterman score: 385;    33.6% identity in 247 aa overlap

                                    10          20        30        40
KYBOA                         CGVPAIQPVLSGLSR--IVNGEEAVPGSWPWQVSLQDKTGFHFCGGSLINE
                                : ..::: .. .   .:.: .:. : .:..: :.   :   :::.:  .
TRSMG  MKHFLRALKRCSVAVATVAIAVVGLQPVTASAAPNPVVGGTRAAQGEFPFMVRLS--MG---CGGALYAQ
               10        20        30        40        50             60

      50            60        70        80        90       100       110
KYBOA  NWVVTAAHC-----GVTTSDVVVAGEFDQGSSSEKIQKLKIAKVFKNSKYNSLTINNDITLLKLSTAASFS
       . :.::::::    : .:: ....:  :  :::    :.. .::..   ::.   ..: .:.:: :  ..
TRSMG  DIVLTAAHCVSGSGNNTSITATGGVVDLQSSSA--VKVRSTKVLQAPGYNGT--GKDWALIKL--AQPIN
          70        80        90         100       110         120

         120       130       140       150       160       170       180
KYBOA  QTVSAVCLPSASDDFAAGTTCVTTGWGLTRYTNANTPDRLQQASLPLLSNTNCKKYWGTK-IKDAMICAG
       : .  .   .:   .  ::  :. ::: .:  ...    : .:..:..:.. :.. .:.. . .  ::::
TRSMG  QPTLKIATTTA---YNQGTFTVA-GWGANR-EGGSQQRYLLKANVPFVSDAACRSAYGNELVAICAG
     130       140           150       160       170       180       190

             190       200       210       220       230       240
KYBOA  ---ASGVSSCMGDSGGPLVCKKNG-AWTLVGIVSWGSSTCSTSTPGVYARVTALVNWVQQTLAAN
          ..::..:.:::::::.  : :.  :  :::::::  .    . ::::..:..... .
TRSMG  YPDTGGVDTCQGDSGGPMFRKDNADEWIQVGIVSWGYGCARPGYPGVYTEVSTFASAIASAARTL
          200       210       220       230       240       250
```

Figure 21: Serine protease alignments

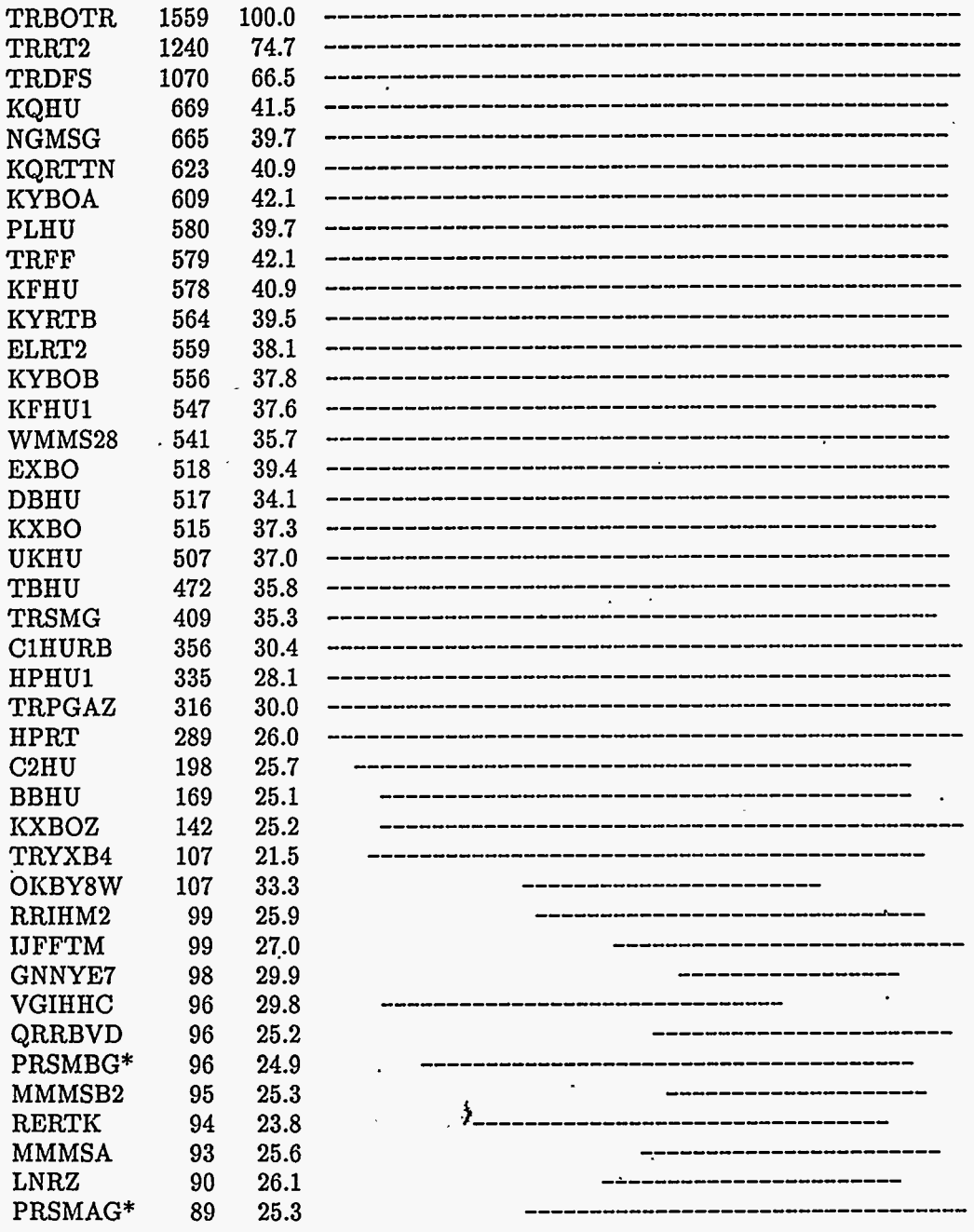


Table 14: Glutathione S-transferases

The best scores are:		s-w	Z-score	E(43470)
GTB1_MOUSE	Glutathione S-transferase GT8.7	1490	2006.4	0
GTB1_RAT	Glutathione S-transferase YB1	1406	1892.9	0
GTM1_HUMAN	Glutathione S-transferase	1235	1661.9	0
GT2_CHICK	Glutathione S-transferase 2	954	1282.1	0
GTP_MOUSE	Glutathione S-transferase P	361	481.2	2.3×10^{-20}
GTA2_MOUSE	Glutathione S-transferase Ya	229	302.2	2.2×10^{-10}
SC2_OCTDO	S-crystallin 2 (OL2).	224	297.2	4.2×10^{-10}
GTA1_MOUSE	Glutathione S-transferase GT41A	218	287.4	1.5×10^{-9}
GTC_MOUSE	Glutathione S-transferase Yc	215	283.4	2.4×10^{-9}
GTH1_HUMAN	Glutathione S-transferase A1-1	206	271.2	1.2×10^{-8}
GT28_SCHHA	Glutathione S-transferase 28 kd	203	267.6	1.8×10^{-8}
GT5A_MOUSE	Glutathione S-transferase GST 5.7	183	240.1	6.3×10^{-7}
GT28_SCHJA	Glutathione S-transferase 28 kd	169	221.9	6.4×10^{-6}
GT2_DROME	Glutathione S-transferase 2	164	213.4	2.0×10^{-5}
SC1_OCTVU	S-crystallin 1.	159	209.0	3.3×10^{-5}
GTAC_CHICK	Glutathione S-transferase, CL-3.	144	187.1	0.00056
SC18_OMMSL	S-crystallin SL18.	131	163.0	0.012
GT1_MUSDO	Glutathione S-transferase 1	122	158.3	0.023
GT1_MAIZE	Glutathione S-transferase I	120	155.3	0.033
ARP_TOBAC	Auxin-regulated protein	117	151.0	0.058
GT32_MAIZE	Glutathione S-transferase III	115	148.2	0.082
GT1_DROME	Glutathione S-transferase 1-1	100	128.5	1.0
GT1_WHEAT	Glutathione S-transferase 1	98	124.9	1.6
GT_PROMI	Glutathione S-transferase GST-6.0	97	124.7	1.7
DCMA_METSP	Dichloromethane dehalogenase	98	122.7	2.2
GTY2_ISSOR	Glutathione S-transferase Y-2	94	121.3	2.6
ARP2_TOBAC	Auxin-induced PGNT35/PCNT111.	93	118.4	3.7
GTT1_RAT	Glutathione S-transferase 5	93	117.8	4.1
MOD5_YEAST	tRNA isopentenyltransferase	100	117.2	4.4
GT2_WHEAT	Glutathione S-transferase 2	92	114.5	6.2
MYSP_MOUSE	Myosin heavy chain, skeletal	81	113.5	7.0
LIGE_PSEPA	β-etherase	91	113.5	7.0
YFHE_ECOLI	hypothetical 20.1 kd protein in HSCA	86	113.5	7.1
EF1G_HUMAN	Elongation factor 1γ (EF-1γ).	94	113.3	7.2
GT_ECOLI	Glutathione S-transferase	88	112.7	7.9
ABF2_YEAST	ARS-binding factor 2 precursor	87	112.2	8.4
KKQ1_YEAST	Probable ser/thr-protein kinase	92	110.7	10.1
EF1G_RABIT	Elongation factor 1γ (EF-1γ).	92	110.6	10.2
ARP3_TOBAC	Auxin-induced PCNT103.	87	110.3	10.6
CYAA_BACAN	Calmodulin-sens. adenylate cyclase	96	110.2	10.7
YJJV_ECOLI	hypoth. 23.7 kd protein	86	110.0	11.1

All of the unitalicized sequences are known to be members of the glutathione transferase family.

4.2 Glutathione S-transferases

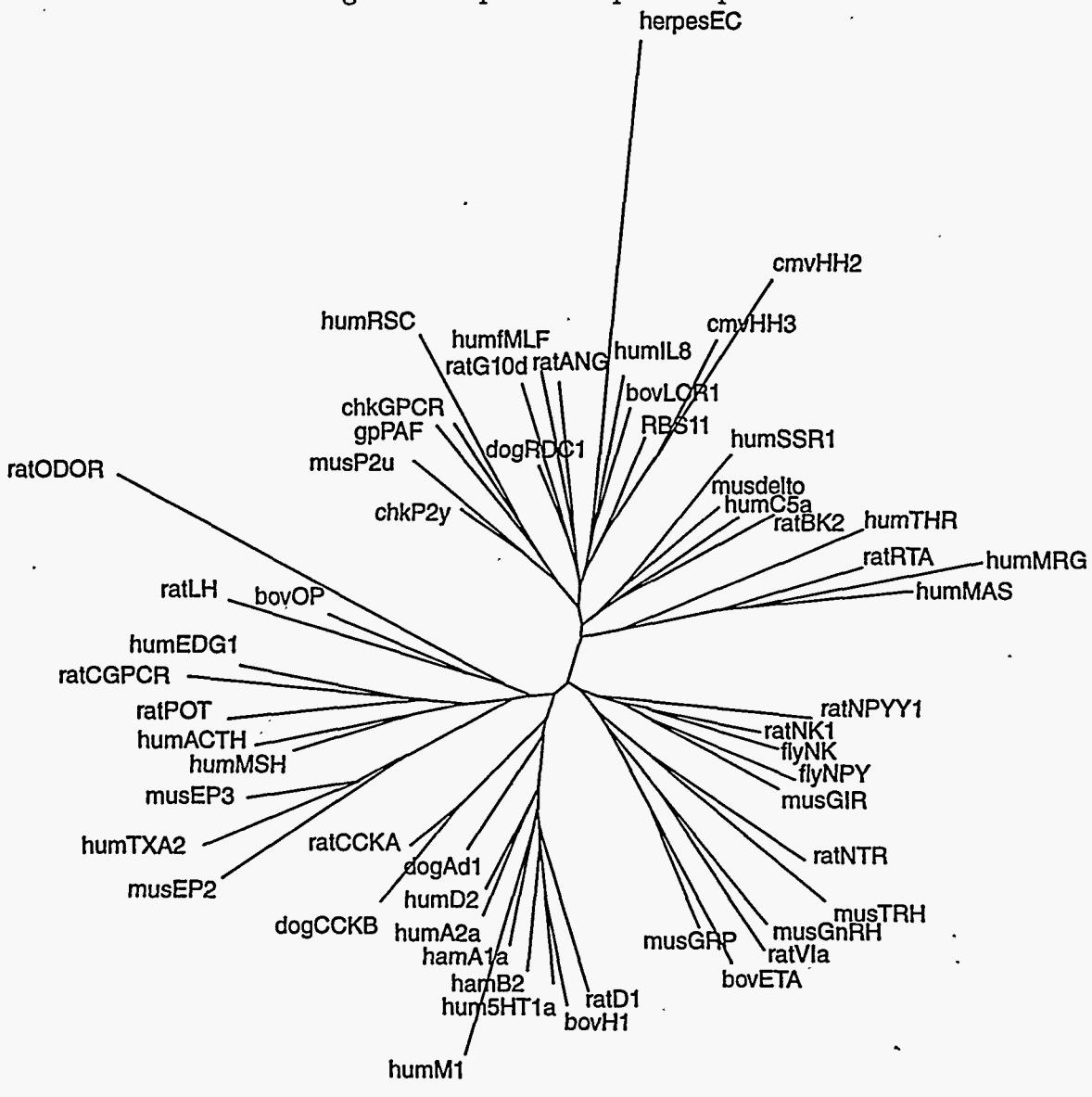
The glutathione transferase family of enzymes is a very diverse family of proteins found, in various forms, in animals, plants, and prokaryotes. Fortunately, many of the members of this family have a common enzyme activity so that they can be recognized by name. Table 14 shows that for this family, there are many homologues that do not show significant similarity when the database is searched with a single query sequence.

Frequently, clear identification of a distant homology will require several database searches, with either different algorithms or additional query sequences. For example, in Table 14, one might wish to test the possibility that glutathione S-transferases shares homology with elongation factors, which are among the high scoring sequences. The result of a search using `EF1G_HUMAN` is shown in Table 15. Here, there is a clear relationship between this elongation factor and the class-theta glutathione transferases. An additional search with a class-theta sequence reveals the most distant relationships in this family more clearly.

Table 15: Glutathione Transferase Homology with EF1γ

The best scores are:		s-w	Z-score	E(43470)
EF1G_HUMAN	Elongation factor 1γ (EF-1γ)	2977	3398.2	0
EF1G_XENLA	Elongation factor 1γ (EF-1γ)	2370	2703.1	0
EF1H_YEAST	Elongation factor 1γ 2 (EF-1γ)	769	870.4	0
EF1G_TRYCR	Elongation factor 1γ (EF-1γ)	715	808.6	0
SYV_HUMAN	valyl-tRNA synthetase	440	408.5	2.6×10^{-16}
GT1_MAIZE	Glutathione S-transferase I	222	250.3	1.7×10^{-7}
GT32_MAIZE	Glutathione S-transferase III	193	216.7	1.3×10^{-5}
GT1_WHEAT	Glutathione S-transferase 1	186	208.4	3.7×10^{-5}
GTB_TOBAC	Glutathione S-transferase	184	206.7	4.5×10^{-5}
GTY2_ISSOR	Glutathione S-transferase Y-2	175	197.5	0.00015
GT2_WHEAT	Glutathione S-transferase 2	175	193.5	0.00025
HS26_SOYBN	Heat shock protein 26A.	171	191.3	0.00033
ARP2_TOBAC	Auxin-induced PGNT35/PCNT111	169	189.1	0.00043
ARP1_TOBAC	Auxin-induced PGNT1/PCNT110	166	185.7	0.00067
ARP3_TOBAC	Auxin-induced PCNT103	163	182.3	0.0010
GT1_DROME	Glutathione S-transferase 1-1	162	181.7	0.0012
YIBF_ECOLI	hypoth. 22.6 kd prot.	155	177.6	0.0019
GT1_DROSE	Glutathione S-transferase 1-1	155	174.1	0.0030
GT1_DROYA	Glutathione S-transferase 1-1	154	173.0	0.0034
GT1_DROER	Glutathione S-transferase 1-1	152	170.7	0.0046
DCMA_METSP	Dichloromethane dehalogenase	153	168.4	0.0062
GT1_DROTE	Glutathione S-transferase 1-1	150	168.4	0.0062
PRP1_SOLTU	Pathogenesis-related prot. 1.	147	166.3	0.0081
GT1_MUSDO	Glutathione S-transferase 1	138	154.3	0.04

Figure 22: G-protein-coupled receptors



4.3 G-protein-coupled receptors

The G-protein-coupled receptors (GCRs) are one of the largest known gene families; members of the family transduce signals from light, peptides, cationic amines, lipid mediators, odors, and many more small molecules. An evolutionary tree that summarizes the diversity of this family is shown in Fig. 22. Based on hydrophobicity plots and the structure of bacteriorhodopsin (a protein that does not share significant similarity with members of this family), the GCRs are thought to contain seven transmembrane domains, so that the N-terminus of the proteins is extracellular, while the C-terminus is intracellular.

Because GCRs have transmembrane domains, the highest scoring unrelated sequences

Table 16: GCRs distant from human opsin

The best scores are:		s-w	Z-score	E(43470)
CAR1_DICDI	CYCLIC AMP RECPT. 1	130	162.0	0.014
OLF2_CHICK	OLFACTORY RECPT.-LIKE PROTEIN COR2	129	158.1	0.022
5H2A_CAVPO	5-HYDROXYTRYPTAMINE 2A RECPT.	121	153.7	0.040
CAR3_DICDI	CYCLIC AMP RECPT. 3.	124	152.2	0.049
MAS_HUMAN	MAS PROTO-ONCOGENE.	120	150.2	0.064
OLF4_CHICK	OLFACTORY RECPT.-LIKE COR4.	121	147.9	0.085
OLF5_CHICK	OLFACTORY RECPT.-LIKE COR5.	120	146.6	0.10
OLF1_CHICK	OLFACTORY RECPT.-LIKE COR1.	117	142.4	0.17
PER2_MOUSE	PROSTAGLANDIN E/EP2 RECPT.	121	140.0	0.23
UL33_HCMVA	G-PROTEIN COUPLED RECPT. HOMOLOG	117	139.2	0.26
GU58_RAT	POSSIBLE GUSTATORY RECPT.	109	138.2	0.30
CAR2_DICDI	CYCLIC AMP RECPT. 2	111	137.0	0.35
MSHR_MOUSE	MELANOCYTE STIM. HORMONE RECPT.	111	134.9	0.45
MSHR_HUMAN	MELANOCYTE STIM. HORMONE RECPT.	111	134.8	0.46
LIVM_ECOLI	BRANCHED-CHAIN AMINO ACID	109	133.3	0.55
APPC_ECOLI	PROB.CYTOCHROME OXIDASE	110	133.1	0.57
BIOX_BACSH	BIOX PROTEIN.	102	131.5	0.69
RTA_RAT	PROB. G PROTEIN-COUPLED RECPT. RTA.	109	131.0	0.74
GU45_RAT	POSS. GUSTATORY RECPT. PTE45	102	128.8	0.99
AROP_ECOLI	AROMATIC AMINO ACID TRANS. PROT. A	106	128.7	1.0
PER1_HUMAN	PROSTAGLANDIN E/EP1 RECPT.	108	127.4	1.2
TCR_STAAU	TETRACYCLINE RESISTANCE PROTEIN.	106	123.9	1.9
OLF4_MOUSE	OLFACTORY RECPT.-LIKE PROTEIN K4	98	123.3	2.0
TCR2_BACSU	TETRACYCLINE RESISTANCE PROTEIN.	106	123.1	2.1
CYOB_ECOLI	CYTOCHROME O UBIQUINOL OXIDASE	104	123.0	2.1

are frequently other membrane proteins. Table 16 lists sequences from Swiss-Prot that have marginally significant matches with a human opsin sequence (there are more than 375 related sequences with expectations ranging from 0–0.01 that are not shown). As with most divergent families, the question becomes, "how do I know that XXX is/is not a GCR?" This is more difficult with the GCRs, because they have long variable length loops in both their extracellular and intracellular domains.

As before, two strategies can be used to evaluate the hypothesis of homology: re-searching the library and statistical significance from shuffling. A search of the Swiss-Prot database reveals that RTA_RAT shares significant similarity ($E(40,000) < 0.01$) with 120 GCRs; 100 more high-ranking scores with less statistical come from GCRs as well. In contrast, the highest ranking scores from the BIOX_BACSH are:

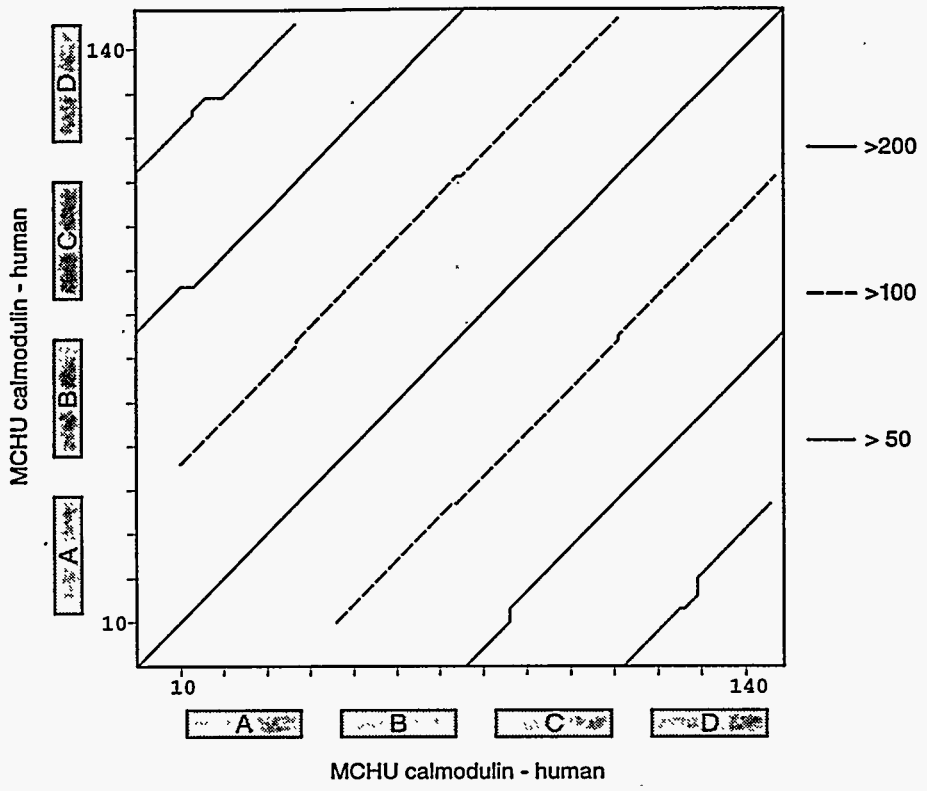
```
The best scores are:                                    s-w Z-score E(43470)
BIOX_BACSH BIOX PROTEIN.                               1029 1305.2       0
POTB_ECOLI SPERMIDINE/PUTRESCINE TRANSPORT SYSTEM       111 138.1 0.3027
PROW_ECOLI GLYCINE BETAINE/L-PROLINE TRANSPORT SYS      112 135.0 0.4493
PIT_ECOLI LOW-AFFINITY INORGANIC PHOSPHATE TRANSPO      113 130.7 0.7754
```

The results from the RTA_RAT and BIOX_BACSH, which show that RTA_RAT is clearly a member of the GCR family, contrast with the statistical significance calculated with the PRSS program. Comparing the OOHU with RTA_RAT score with the distribution of scores calculated after shuffling RTA_RAT 1000 times with a local window of 20 suggests that the unshuffled score (109) is expected 4.6 times in 1000 shuffles. In contrast, the BIOX_BACSU score is expected only 0.8 times in 100 shuffles. From this perspective, the BIOX_BACSHU score is more significant, but, in fact, neither similarity score is significant. It is not until RTA_RAT is compared with other members of the familied, e.g. the angiotensin, fMet-Leu-Phe, IL8, or somatostatin receptors with E-values from 10^{-11}–10^{-6}, that the relationship is apparent.

Table 3.3 compares the statistical significance inferred from database searches with those determined by Monte-Carlo shuffling. As expected, the significance of the scores when compared with locally (window) shuffled sequences is 10-fold lower than the comparison with globally shuffled scores. It is unclear how to compare the expectation from shuffles with the expectation from a search. In the table, the expectation from a search of a 43,000 entry library is compared to the expectation from 1,000 shuffles. For global shuffles, the expectations are quite comparable while local shuffles are more conservative, yet all but one of the similarity scores judged significant from the database search are still significant when compared with the local-shuffle distribution.

Nevertheless, these examples show both that current statistical models for the similarity scores of unrelated sequences are quite accurate, but also that homologous sequences frequently do not share significant pair-wise similarity scores. Thus, a lack of statistical significance cannot be used to infer non-homology, but strong statistical significance is a good indicator of common ancestry.

Figure 23: Internal duplications in calmodulin



5 Repeated structures in proteins

So far, we have focussed on the identification and statistics of the single most significant similarity score shared by two sequences. As can be seen in Fig. 13B, however, there are frequently several non-overlapping local alignments with optimal similarity scores. In addition, there can be non-overlapping sub-optimal alignments with significant scores that can be used to infer the duplication events that gave rise to the protein sequence. An algorithm for the best N non-overlapping local alignments was described by (Waterman & Eggert, 1987).

Figs. 23 and 24 show a graphical plot of the local similarities within the calmodulin calcium binding protein. Calmodulin contains four EF-hand Ca^{+}-binding domains that are well conserved. The highest scoring alignment in Fig. 24 aligns domains A-B with C-D; the second highest aligns A-B-C with B-C-D; the third aligns A with D.

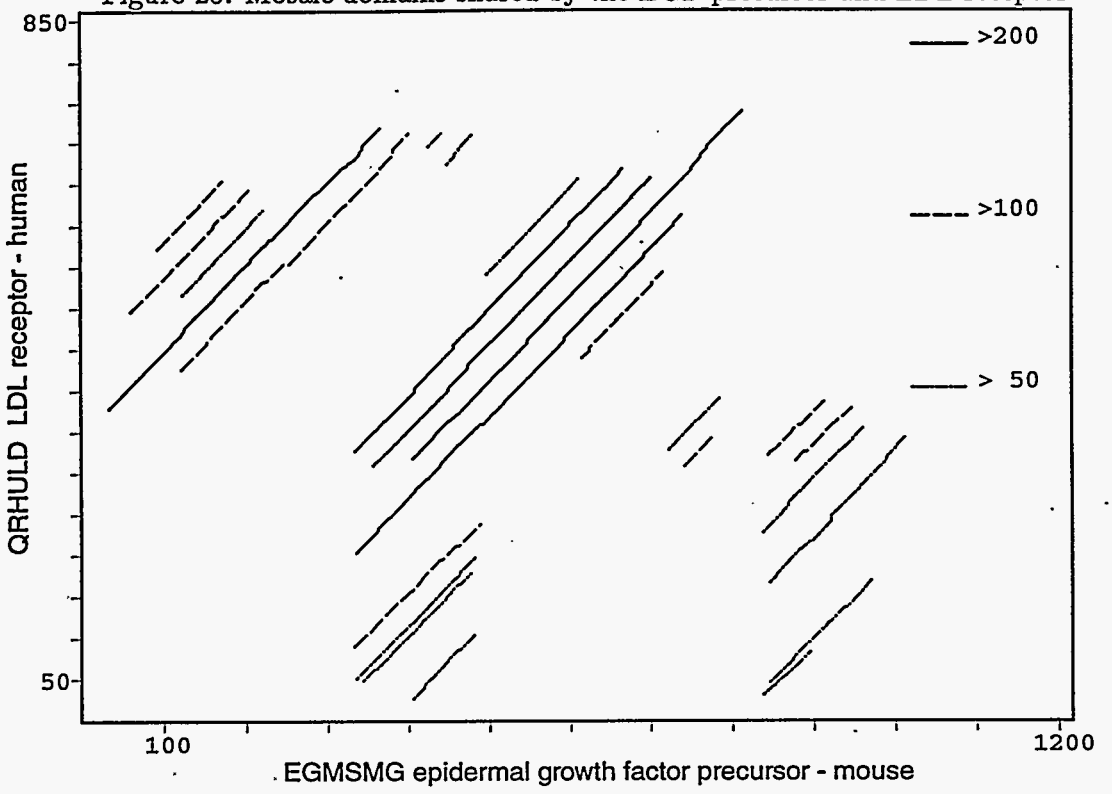
A similar pattern of local similarity can be seen in Fig. 25, which shows the mosaic relationship between the EGF-precursor and the LDL-receptor.

Some non-homologous structures, particularly proteins containing the coiled-coil structure, have a periodic structure that is easily seen in local similarity plots. Fig. 26 shows local similarities in tropomyosin. All the alignments shown have local similarity scores greater

Figure 24: Calmodulin internal alignments

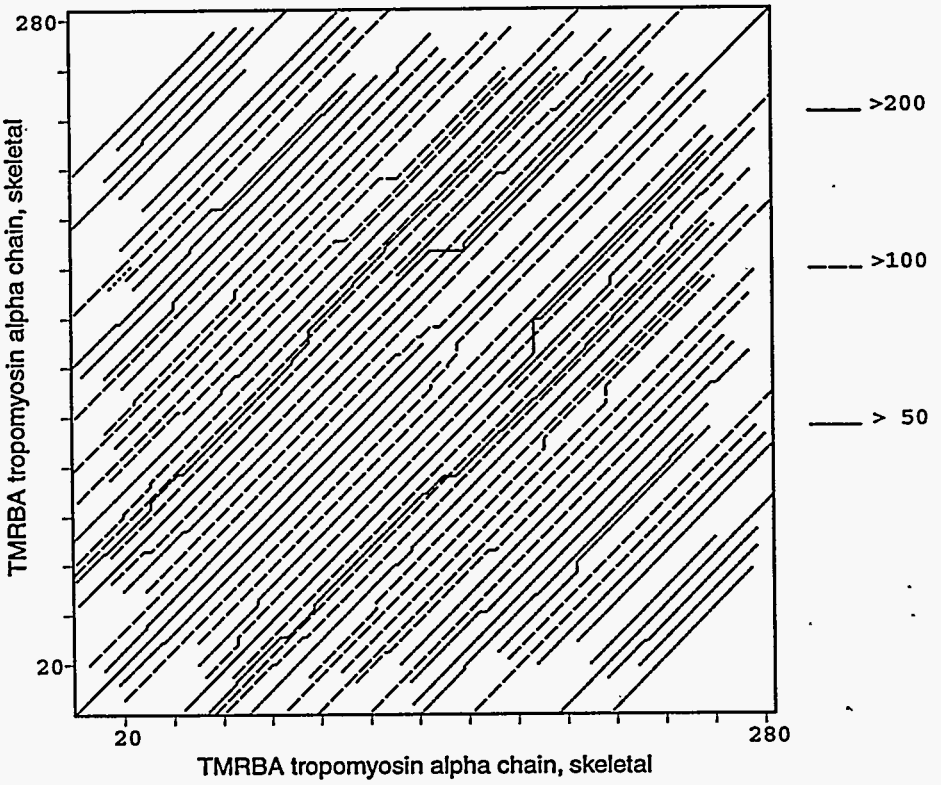
```
 Comparison of:
(A) >MCHU - Calmodulin - Human, rabbit, bovine, rat,   - 148 aa
(B) >MCHU - Calmodulin - Human, rabbit, bovine, rat,   - 148 aa
 using matrix file: BLOSUM50, gap penalties: -14/-4

  47.7% identity in 65 aa overlap; score:  214

               20        30        40        50        60        70
MCHU   EFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARK
       :..::: .::::::.: :.. ::  :: .::.. :. :...:: :.: ::.: ... ::. ::. :
MCHU   EIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAK
            90       100       110       120       130       140
----------
  32.4% identity in 102 aa overlap; score:  177

      10        20        30        40        50        60        70
MCHU   AEFKEAFSLFDKDGDGTITTKELGTVM-RSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKD
       ::... ..  : ::.:::   :. :.: :.. .. .: :... .   : :::: :.  :.  .:. .. .
MCHU   AELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMT-NLGE
          50        60        70        80        90       100       110

       80        90       100       110
MCHU   TDSEEEIREAFRVFDKDGNGYISAAELRHVMT
         ..::. : .:  : ::.: ..  :. ..::
MCHU   KLTDEEVDEMIREADIDGDGQVNYEEFVQMMT
          120       130       140
----------
  36.1% identity in 36 aa overlap; score:   55

             10        20        30
MCHU   DQLTEEQIAEF-KEAFSLFDKDGDGTITTKELGTVM
       ..::.:.. :. .::    : :::: .. .:.  .:
MCHU   EKLTDEEVDEMIREA----DIDGDGQVNYEEFVQMM
           120           130       140
----------
  40.0% identity in 20 aa overlap; score:   53

       70        80
MCHU   LTMMARKMKDTDSEEEIREA
       .: ...:. : . .: ::::
MCHU   MTNLGEKLTDEEVDEMIREA
      110       120
----------
```

Figure 25: Mosaic domains shared by the EGF-precursor and LDL-receptor



than 120, and each would be significant in a conventional database search.

Figure 26: Coiled-coil structures share local similarity



6 Summary

Protein sequence comparison is the most powerful tool available today for inferring structure and function from sequence because of the constraints of protein evolution—a protein fold into a functional structure. Protein sequence similarity can routinely be used to infer relationships between proteins that last shared a common ancestor 1–2.5 billion years ago. Our ability to identify distantly related proteins has improved over the past five years with the development of accurate statistical estimates, which have provided better normalization methods, and with the use of optimized scoring parameters. In using sequence similarity to infer homology, one should remember:

1. Always compare protein sequences if the genes encode proteins. Protein sequence comparison will typically double the look back time over DNA sequence comparison.

2. While most sequences that share statistically significant similarity are homologous, many distantly related homologous sequences do not share significant homology. (Low complexity regions display significant similarity in the absense of homology). Homologous sequences are usually similar over an entire sequence or domain. Matches that are more than 50% identical in a 20–40 amino acid region occur frequently by chance.

3. Homologous sequences share a common ancestor, and thus a common protein fold. Depending on the evolutionary distance and divergence path, two or more homologous sequences may have very few absolutely conserved residues. However, if homology has been inferred between **A** and **B**, between **B** and **C**, and between **C** and **D**, **A** and **D** must be homologous, even if they share no significant similarity.

4. Similarity searching techniques can be improved either by increasing the ability of a method to recognize distantly related sequences—increased sensitivity—or by lowering scores for unrelated sequences—increased selectivity. Since there are generally 1000-times more unrelated than related sequences in a sequence database, improvements that reduce the scores of unrelated sequences can have dramatic effects. The most dramatic improvements in comparison methods recently have used this approach.

References

Altschul, S. F. (1991). Amino acid substitution matrices from an information theoretic perspective. *J. Mol. Biol.* **219**, 555–565.

Altschul, S. F. (1993). A protein alignment scoring system sensitive at all evolutionary distances. *J. Mol. Evol.* **36**, 290–300.

Altschul, S. F., Boguski, M. S., Gish, W. & Wootton, J. C. (1994). Issues in searching molecular sequence databases. *Nature Genet.* **6**, 119–129.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). A basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Collins, J. F., Coulson, A. F. W. & Lyall, A. (1988). The significance of protein sequence similarities. *Comp. Appl. Biosci.* 4, 67–71.

Dayhoff, M., Schwartz, R. M. & Orcutt, B. C. (1978). A model of evolutionary change in proteins. In *Atlas of Protein Sequence and Structure*, (Dayhoff, M., ed.), vol. 5, supplement 3, pp. 345–352. National Biomedical Research Foundation Silver Spring, MD.

Doolittle, R. F., Feng, D. F., Johnson, M. S. & McClure, M. A. (1986). Relationships of human protein sequences to those of other organisms. *Cold Spring Harb. Symp. Quant. Biol.* **51**, 447–455.

Gilbert, W. & Glynias, M. (1993). On the ancient nature of introns. *Gene,* **135**, 137–144.

Henikoff, S. & Henikoff, J. G. (1992). Amino acid substitutions matrices from protein blocks. *Proc. Natl. Acad. Sci. USA,* **89**, 10915–10919.

Henikoff, S. & Henikoff, J. G. (1993). Performance evalutation of amino-acid substitution matrices. *Proteins,* **17**, 49–61.

Johnson, M. S. & Overington, J. P. (1993). A structural basis for sequence comparisons. an evaluation of scoring methodologies. *J. Mol. Biol.* **233**, 716–738.

Jones, D. T., Taylor, W. R. & Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *Comp. Appl. Biosci.* **8**, 275–282.

Karlin, S. & Altschul, S. F. (1990). Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. *Proc. Natl. Acad. Sci. USA,* **87**, 2264–2268.

Karlin, S. & Altschul, S. F. (1993). Applications and statistics for multiple high-scoring segments in molecular sequences. *Proc. Natl. Acad. Sci USA,* **90**, 5873–5877.

Mott, R. (1992). Maximum-likelihood estimation of the statistical distribution of smith-waterman local sequence similarity scores. *Bull. Math. Biol.* **54**, 59–75.

Needleman, S. & Wunsch, C. (1970). A general method applicable to the search for similarities in the amino acid sequences of two proteins. *J. Mol. Biol.* 48, 444–453.

Nei, M. (1987). *Molecular Evolutionary Genetics.* Columbia Univ. Press, New York, NY.

Pearson, W. R. (1995). Comparison of methods for searching protein sequence databases. *Prot. Sci.* 4, 1145–1160.

Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA,* 85, 2444–2448.

Smith, T. F. & Waterman, M. S. (1981). Identification of common molecular subsequences. *J. Mol. Biol.* 147, 195–197.

Stoltzfus, A., Spencer, D. F., Zuker, M., Logsdon, J. M. & Doolittle, W. F. (1994). Testing the intron theory of genes: the evidence from protein structure. *Science,* 265, 202–207.

Waterman, M. S. & Eggert, M. (1987). A new algorithm for best subsequences alignment with application to tRNA-rRNA comparisons. *J. Mol. Biol.* 197, 723–728.

7 Suggested Reading

7.1 General Protein evolution

R. F. Doolittle, D. F. Feng, M. S. Johnson, and M. A. McClure. Relationships of human protein sequences to those of other organisms. *Cold Spring Harb. Symp. Quant. Biol.*, 51:447–455, 1986.

P. Green, D. Lipman, L. Hillier, R. Waterston, D. States, and J. M. Claverie. Ancient conserved regions in new gene sequences and the protein databases. *Science*, 259:1711–1716, 1993.

7.1.1 Introns Early/Late

W. Gilbert and M. Glynias. On the ancient nature of introns. *Gene*, 135:137–144, 1993.

A. Stoltzfus, D. F. Spencer, M. Zuker, J. M. Logsdon, and W. F. Doolittle. Testing the intron theory of genes: the evidence from protein structure. *Science*, 265:202–207, 1994.

7.2 Alignment methods

7.2.1 Algorithms

S. Needleman and C. Wunsch. A general method applicable to the search for similarities in the amino acid sequences of two proteins. *J. Mol. Biol.*, 48:444–453, 1970.

T. F. Smith and M. S. Waterman. Identification of common molecular subsequences. *J. Mol. Biol.*, 147:195–197, 1981.

W. R. Pearson and W. Miller. Dynamic programming algorithms for biological sequence comparison. In L. Brand and M. L. Johnson, editors, *Meth. Enz.*, volume 210, pages 575–601. Academic Press, San Diego, 1992.

D. J. Lipman and W. R. Pearson. Rapid and sensitive protein similarity searches. *Science*, 227:1435–1441, 1985.

W. R. Pearson and D. J. Lipman. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA*, 85:2444–2448, 1988.

W. R. Pearson. Rapid and sensitive sequence comparison with FASTP and FASTA. In R. F. Doolittle, editor, *Meth. Enz.*, volume 183, pages 63–98. Academic Press, San Diego, 1990.

S. F. Altschul, W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. A basic local alignment search tool. *J. Mol. Biol.*, 215:403–410, 1990.

W. R. Pearson. Searching protein sequence libraries: Comparison of the sensitivity and selectivity of the Smith-Waterman and FASTA algorithms. *Genomics*, 11:635–650, 1991.

W. R. Pearson. Comparison of methods for searching protein sequence databases. *Prot. Sci.*, 4:1145–1160, 1995.

7.2.2 Scoring methods

M. Dayhoff, R. M. Schwartz, and B. C. Orcutt. A model of evolutionary change in proteins. In M. Dayhoff, editor, *Atlas of Protein Sequence and Structure*, volume 5, supplement 3, pages 345–352. National Biomedical Research Foundation, Silver Spring, MD, 1978.

S. F. Altschul. Amino acid substitution matrices from an information theoretic perspective. *J. Mol. Biol.*, 219:555–565, 1991.

D. T. Jones, W. R. Taylor, and J. M. Thornton. The rapid generation of mutation data matrices from protein sequences. *Comp. Appl. Biosci.*, 8:275–282, 1992.

S. Henikoff and J. G. Henikoff. Performance evalutation of amino-acid substitution matrices. *Proteins*, 17:49–61, 1993.

7.3 Evaluating matches - statistics of similarity scores

R. F. Doolittle. Similar amino acid sequences: chance or common ancestry? *Science*, 214:149–159, 1981.

W. R. Pearson. Identifying distantly related protein sequences. *Cur. Opinion in Struct. Biol.*, 1:321–326, 1991.

S. Karlin, P. Bucher, V. Brendel, and S. F. Altschul. Statistical methods and insights for protein and DNA sequences. *Ann. Rev. of Biophys. Biophys. Chem.*, 20:175–203, 1991.

S. F. Altschul, M. S. Boguski, W. Gish, and J. C. Wootton. Issues in searching molecular sequence databases. *Nature Genet.*, 6:119–129, 1994.

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