New algorithms and methods for protein and DNA sequence comparison

James Crook

A Thesis Presented for
the Degree of
Ph.D.

Biocomputing Research Unit,
Department of Molecular Biology,
University of Edinburgh.

May 1991.

Abstract

International biological sequence databases hold information about protein and DNA molecules. The molecules are represented by sequences of characters. In analysis of this data algorithms for comparing the character sequences play a central role. Comparisons can be made using dynamic programming techniques to determine the score of optimal sequence alignments. Such methods are particularly popular with molecular biologists for they accommodate the kinds of differences which actually occur in the sequences of related molecules.

Sequence alignments are normally scored using score tables based on an evolutionary model. The derivation of these score tables is re-examined and a formula giving an analytic counterpart to an empirical method for assessment of a score table's discriminating power is found. Use of the formula to derive alternative protein similarity scoring tables is discussed.

A new approach to tackling the heavy computational demands of the dynamic programming algorithm is described: intensive optimisation of a microcomputer implementation. This provides an alternative to implementations which use parallel computers for searching protein databases. This thesis also describes how other implementational problems were tackled in order to make more effective use of the serial comparison software.

The new software permitted comparison by optimal alignment of 32,000,000 pairs of sequences from a protein database using widely available and inexpensive hardware. The results from this search were then reorganised to facilitate the finding of previously unseen similarities. Software tools were written to assist with the analysis including software to align sequence families.

From the results of this work, nine similarities are presented which do not appear to have been previously noted. The examples illustrate factors that are important in assessing similarities with scores close to the boundaries of significance. The similarities presented are of particular interest because of the biological functions they relate.

One software tool developed for the sequence analysis work was a new multiple sequence alignment editor and sequence aligner, "Medal". Lessons from its use on real sequence data lead to a modification to the original comparison method to accommodate local variations in sequence similarity.

Consideration is given to parallelisation of this modification and of the methods used to obtain speed in the serial software. Alternatives are suggested. The suggested parallel method to cope with variations in sequence similarity requires two interdependent sequence comparisons. A serial program using three interdependent comparisons is demonstrated and shows the feasibility of multiple interdependent comparisons. Examples show how this new program, "Fradho", can compare DNA sequences to protein sequences accommodating frameshifts.

Acknowledgements

Firstly I thank my two supervisors. I particularly want to thank Professor Sidney Michaelson (Computer Science) for his constructive criticism combined with lively interest and encouragement. I wish he were still alive and that we could talk about shared interests in Computer Science, Molecular Biology and Mathematics. I also thank Dr John Collins (Molecular Biology) and I thank the Science and Engineering Research Council for their funding.

There are many other people I want to thank too. I thank Andrew Lyall, whose achievements in biological sequence analysis and enthusiasm encouraged me to join in too; Duncan Rouch, who showed me the importance of comparative testing; Dr Andrew Coulson for instruction in "O" and help with laser printer problems; Ian Campbell for attempting, with intermittent success, to repair the jinxed IBM PC clone; for making the computing unit more human, Carol Crawley, Tom Smith, Alok Kumar, Steven Hayward and Sarah (SJ) McQuay whom I also thank for comments on the draft; Adelaide Carpenter for showing me how to copy edit on an early draft of Chapter 8; the excellent staff at the Darwin library for all their help; Richard Hayward for comments on *S. aureus* protein-A; Stuart Moodie for drawing my attention to a paper on the p53 antigen; Roger Slee for providing dodgy DNA data at an early stage of sequencing; Michelle Ginsburg for the viral subunit sequences to align; Gill Cowan for explaining the restriction enzyme identification problem and Mike Holmes and Peter Cleat in the computing support for biologists group. Finally I want to thank my parents, sister and my girlfriend Bobby for all their encouragement. Any mistakes in this thesis are of course my own, alas, in this instance blaming the computer does not help me much.

Declaration

The following declaration is made in accordance with paragraph 3.4.7 of the University of Edinburgh regulations governing the submission of theses.

I declare that this thesis has been composed by me and that all the work described in it is my own unless clearly indicated otherwise.

James Crook
May 1991.
Edinburgh.

Contents

Chapter 1: Introduction 1

Biological sequence data 1

Software for sequence analysis 13

Importance of sequence comparison 13

Concluding remarks 16

Chapter 2: Comparison Methods and the NWS Algorithms 17

Word based comparison 17

Estimating likelihoods of chance matching 18

Alphabet reduction 20

Scoring amino acid similarity 21

Comparison by alignment 23

Chapter 3: Measuring Similarity 29

The Dayhoff model 30

Assumptions of the model 31

Justification for method for deriving 1 PAM table 38

Explanation for symmetry of A' 39

Discrimination of similarity scoring tables 40

Other amino acid similarity scoring tables 46

Chapter 4: Rapid and Sensitive Database Searching 47

Sensitivity and selectivity 49

Computational cost 49

Database reduction 51

Approximate methods for comparison 54

Theoretical advances in comparison algorithms 56

Concluding remarks 57

Chapter 5: Techniques to Get More from Machines 58

Type III comparison (speed) 58

Path reconstruction (memory space) 61

Database compression (disk space) 64

Tripeptide matching (speed) 66

Annotation browser (portability) 67

Optimisations to "Prosrch" 68

Concluding remarks 70

Chapter 6: Multiple Sequence Alignment **71**
Uses of multiple alignments 72
Sequence editors 74
Automatic alignment methods 82
Use of the new computer tools 88
Concluding remarks 91

Chapter 7: Comprehensive Database Analysis **93**
Organisation of sequence data 93
Methods and data used in comprehensive search 94
Reduction of the similarity data 97
Tree formation 100
Examining results 101
Concluding remarks 109

Chapter 8: Twilight Zone Similarities **111**
Scores to significances 112
Repetitive sequence problem: Protein-A 114
Weak local constraints 116
Viral repetitive proteins 117
Local similarity and local structure 118
Multiple matching: An AMP binding pattern? 119
Conserved cysteines: Two plasma proteins 122
Similarities across taxonomic boundaries 123
Two pathogenesis related proteins 124
Protein formation and folding 125
Arginosuccinate and two viral proteins 126
Phosphoenolpyruvate-regulated sugar transport 127
Concluding remarks 127

Chapter 9: NWS variants **129**
Coding regions and frameshifts 129
"Fradho" 131
Variations in noise 140
Mixed noise Type III comparison 142

Addendum: The "Blast" algorithm **144**
"Blast's" method for finding word-pairs 145
Validity of seed based matching 146

Appendix 1: Tests of some ideas for new software **147**
Introduction 147
Sequence retrieval 147
Dotplots 149
Identification of a target pattern 151

Appendix 2: Methods for fast serial type III comparison **154**

Appendix 3: Conditional deferred assignment **161**

Appendix 4: Potential for optimisations to "Prosrch" **163**

Abbreviations **168**

References **170**

Chapter 1: Introduction

This interdisciplinary thesis seeks to improve the ways in which Information Technology is applied to the analysis of biological sequence data. This is simultaneously a theoretical and a practical problem. The approach taken in this work is a pragmatic one. Both the nature of the data being studied and practical computational issues need to be considered. We start by describing the nature of biological sequence data, how this information represents biological molecules and how these molecules relate to genetic information, the information held in cells that is passed on when cells divide.

Biological sequence data

Polymeric macromolecules synthesised within cells are fundamental to the chemical processes on which all life depends. During the last two decades molecular biologists have characterised many thousands of these macromolecules. The chemical structures they have determined are conveniently represented by sequences of characters. These sequences, the biological sequence data, have been collected on an international scale to make biological sequence databases (Barker *et al.*, 1990; Kahn & Cameron, 1990; Burks *et al.*, 1990).

Two classes of macromolecule are represented in the databases. One class is the protein molecules. Proteins are polymers of amino acids. The other class is the deoxyribonucleic acid (DNA) molecules. DNAs are polymers of nucleotide monomers. Structural and other information about the component monomers of proteins and DNAs is given, mostly in diagramatic form, in figures 1.1 to 1.4. These diagrams are explained in greater detail in later sections of this chapter.

Both proteins and DNAs are linear polymers. The order of characters in the sequences representing a protein or DNA molecule corresponds with the order in which monomers form the linear polymer chain.

As well as holding protein and DNA sequence data, the databases contain textual information. The textual information describes the known roles of the molecules and gives references to the literature in which the sequence data were reported.

Genetic information

Collecting information about DNAs and proteins is an essential part of the process of investigating how organisms function at the molecular level. The chemical reactions in a cell form a highly complex organised system. Multi-stage metabolic pathways convert chemical resources available to a cell into forms that meet the cell's current needs. Protein molecules are crucial to such pathways. In a typical pathway, each reaction is catalysed by a specific protein. Genetic information specifies the kinds of protein synthesised by the cell and hence it determines the metabolic pathways.

The structure of a protein determines how it interacts with other molecules. Taking a specific example, some chemical groups at the surface of a protein may be arranged so that they readily bind to a comparatively small molecule, adenosine triphosphate (ATP). ATP is an important carrier of readily available energy. By specifying the chemical structures of proteins synthesised in the cell, genetic information determines how chemical energy is used.

Like chemical energy, genetic information is vital to the cell. Accurate information is essential to the correct functioning of cellular chemistry. A bacterial cell such as *Escherichia coli* holds genetic information that precisely specifies over 3000 different proteins each of which is formed from, on average, 300 amino acids. Cells also carry information to regulate the synthesis of proteins, to ensure that under differing conditions that the appropriate quantity of each protein is made. Just as there are molecules which carry chemical energy, so too there are molecules which carry genetic information. Molecules of DNA are the primary carriers of genetic information. DNA carries the information which specifies the proteins.

With the discovery of the double helical structure of DNA (Watson & Crick, 1953), the manner in which cells pass on genetic information to their progeny became clear for the first time. The paired structure of DNA suggests a biochemical mechanism for replication of DNA. Chemical processes in the cell make precise copies of DNA molecules thus passing information on to the cell's descendants.

Other achievements fundamental to the discipline of Molecular Biology followed the discovery of DNA's structure. These included development of techniques by Sanger *et al.* (1977), to rapidly determine base sequences of DNA molecules. These techniques make it possible for molecular biologists to read genetic information encoded in DNA. Over the years these techniques have been used extensively and refined. Much molecular biological research can be viewed as experimental work aimed at understanding the function of the data encoded in different DNA molecules.

A landmark achievement in the interpretation of DNA sequence data has been the elucidation of one of the basic information encoding strategies used by DNA, the so called 'genetic code' (Frisch, 1966). Using a table giving the genetic code it is now possible to deduce the sequence of amino acid residues of a protein from the DNA sequence that encodes it.

We now briefly describe the molecules, proteins and DNAs, that are represented by sequence data. We then describe the link between the two classes of molecule. More extensive information can be found in standard texts on Molecular Biology such as Lewin's "Genes" (1990).

DNAs

The now famous DNA double helix consists of a pair of antiparallel complementary strands of deoxyribonucleic acid. Each strand is a linear polymer of thousands of nucleotide monomers. The nucleotides are similar in a sugar-phosphate part which makes the 'backbone' of the strand. They differ in a nitrogenous chemical group called a base. Information is carried by the sequence in which the bases occur along a single strand's length. Four characters c, t, a and g, are used in the sequence representation. They represent the four bases cytosine, thymine, adenine and guanine. The order of characters in a sequence corresponds with the order of chemical groups in one strand.

The bases come in complementary pairs; c pairs with g and t pairs with a. Where one strand has c the complementary strand has g and similarly for each of the other three possibilities. The pairing arises because the individual bases can form stable interactions with their specific partner through weak 'hydrogen bonding'. Hydrogen bonding of bases is shown in figure 1.1. This figure also shows the chemical structure of the four bases. Only one strand of each double-stranded DNA molecule is represented in the databases since the complementary strand can be deduced from its partner. The complementary pairing of two strands is both the basis for the stability of double stranded DNA as a molecule and also the basis of the mechanism of information preserving replication of DNA.

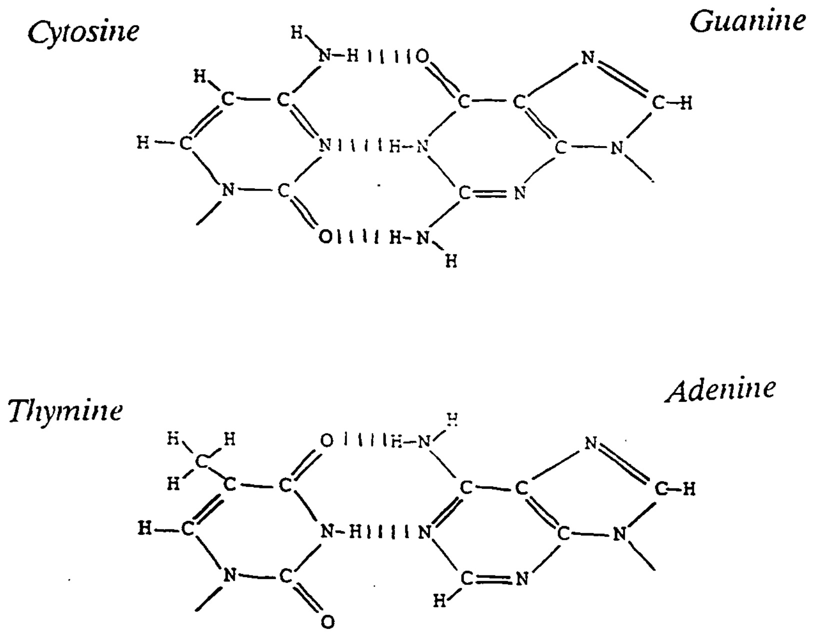


Figure 1.1: Hydrogen bond formation between complementary base pairs.

Proteins

Protein sequences have greater prominence in this work than do DNA sequences. Whereas DNA is the information carrier, proteins are the active expression of this information. Proteins are linear polymers of twenty kinds of amino acid.

Like DNA character sequences, protein character sequences represent the order of simple subunits of a polymeric macromolecule. Protein sequences vary in length from a few tens of characters (e.g. some hormones) to several thousand characters (e.g. viral polyproteins). Each character in the sequence represents an amino acid residue. The term 'residue' is used here to denote the parts of the amino acid left after the polymerisation reaction is complete. In the reaction the carboxyl group of one amino acid reacts with the amino group of the next (figure 1.4). The residue of the amino acid consists of atoms involved in the protein backbone plus a side-chain characteristic of the amino acid. The side-chains are illustrated in figure 1.3. Proteins of around thirty or fewer residues are sometimes referred to as polypeptides, or just peptides, for the bond between residues is known as a peptide bond.

Unlike DNAs, proteins are single stranded polymers. Proteins fold into complex three dimensional structures due to side-chain interactions (Schulz & Schirmer, 1979). Aromatic residues, Phe, Trp, Tyr ; and aliphatic residues, Ile, Ala Leu, Val, have hydrophobic water repelling side-chains (Taylor, 1987a). Protein folding, at least in an aqueous environment, is largely driven by a reduction in energy through hydrophobic residues becoming buried in the core of the structure.

Two particular kinds of region of regular substructure are frequently found in proteins. These are near planar zig-zag substructures (beta sheet), and helical substructures (alpha helix). Both these sub-structures are characterised by a regular pattern of hydrogen bonding involving atoms of the protein backbone. The folded structure adopted by the protein is energetically favourable, that is, slight perturbations of the structure have higher energy and are less stable. The folded structure is essential to the protein's biochemical activity. The regular sub-structures are crucial to the formation and stability of the folded molecule.

Frequently there is biologically significant modification of these basic structures. The folded structure may be further stabilised by the formation of covalent 'disulphide bridges' between cysteine residues in different regions of the sequence that have been brought close together by the folding. Other residues on the surface of the protein may also be chemically modified. However, three dimensional structure is essentially dependent on the sequence.

Amino Acid	Abbr.	Symbol	Frequency
Glycine	Gly	G	0.089
Alanine	Ala	A	0.087
Leucine	Leu	L	0.085
Lysine	Lys	K	0.081
Serine	Ser	S	0.070
Valine	Val	V	0.065
Threonine	Thr	T	0.058
Proline	Pro	P	0.051
Glutamic acid	Glu	E	0.050
Aspartic acid	Asp	D	0.047
Arginine	Arg	R	0.041
Asparagine	Asn	N	0.040
Phenylalanine	Phe	F	0.040
Glutamine	Gln	Q	0.038
Isoleucine	Ile	I	0.037
Histidine	His	H	0.034
Cysteine	Cys	C	0.033
Tyrosine	Tyr	Y	0.030
Methionine	Met	M	0.015
Tryptophan	Trp	W	0.010

Figure 1.2: The amino acids and their standard three letter and one letter abbreviations. The list is in order of abundance, glycine being most abundant and tryptophan being least abundant. After Dayhoff et al. *(1978).*

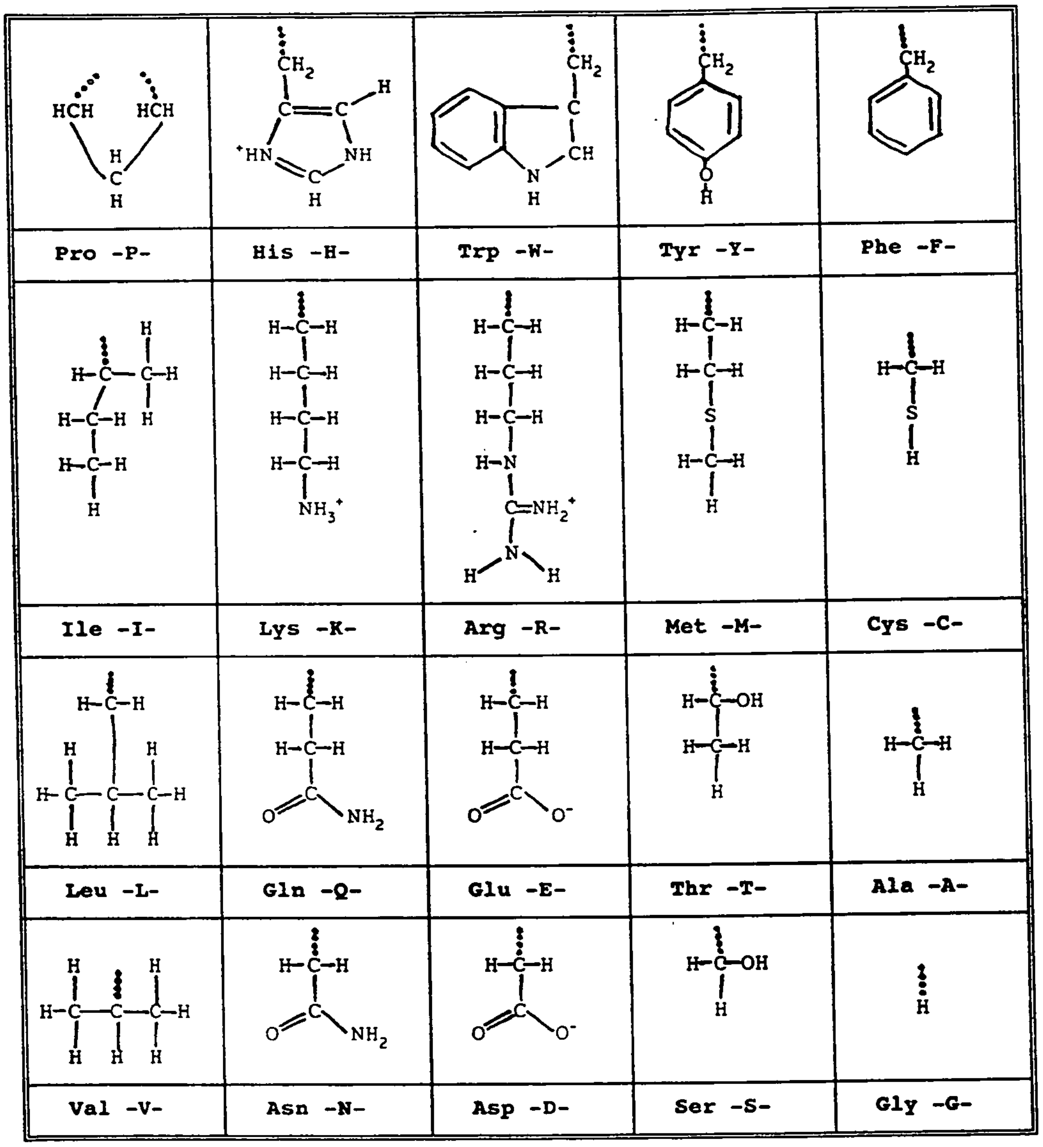


Figure 1.3: The characteristic side chains of different amino acids. These have been arranged in an unconventional way to emphasize some of the similarities and differences. For example, side chains in row 3 differ to those in row 4 only by addition of an extra carbon group. The similarities of individual amino acid types are crucial to comparison of protein sequences with sensitivity.

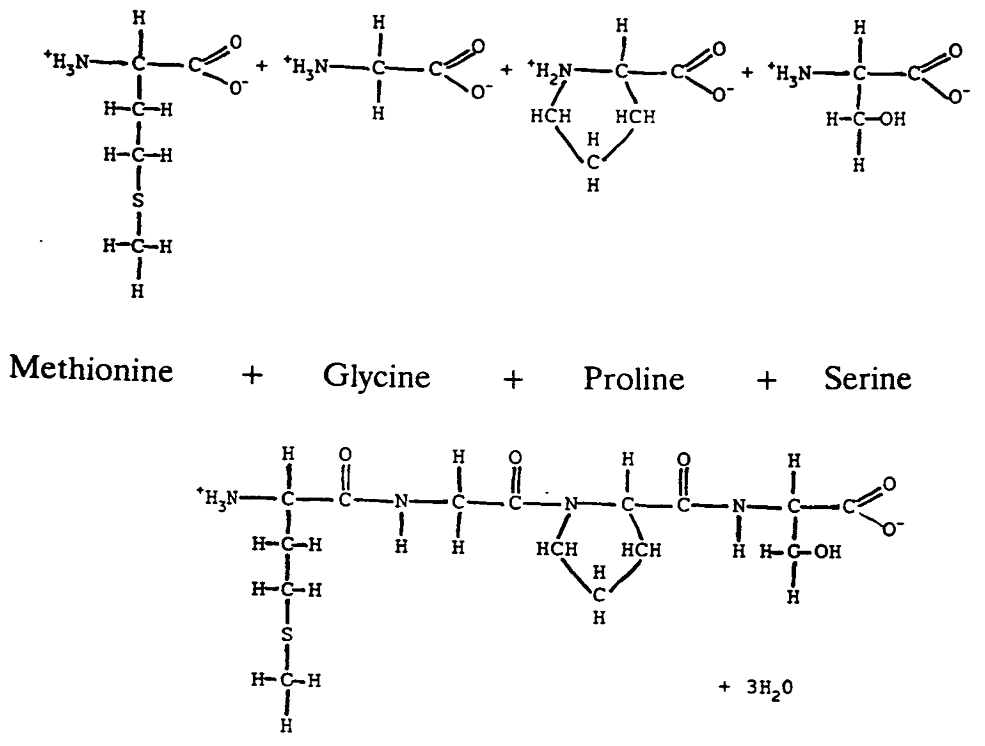


Figure 1.4: Polymerisation of amino acids to form a polypeptide.

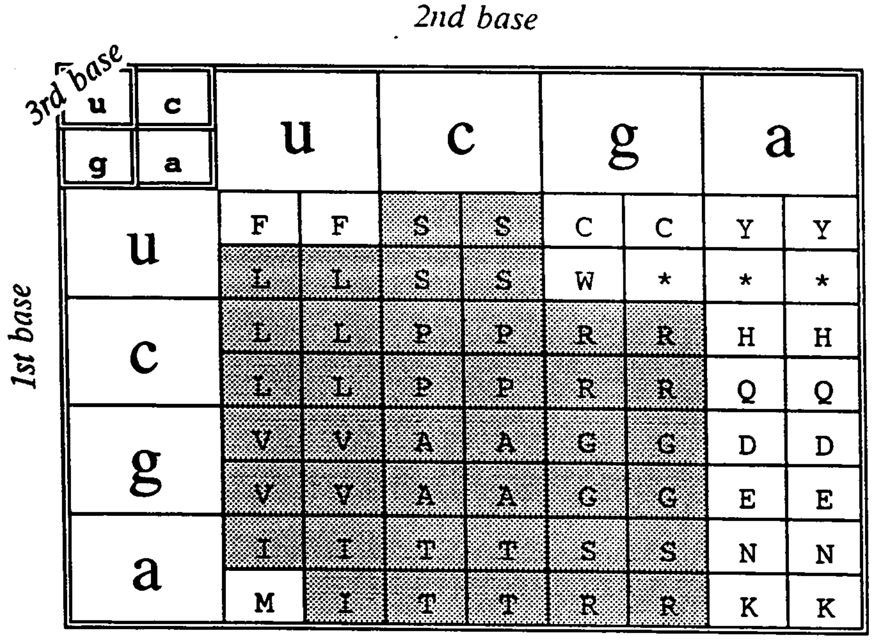
Protein synthesis

The folded proteins have an astonishingly diverse range of functions, structural, regulatory and catalytic. Proteins with catalytic roles are particularly important to cellular chemistry. They are known as 'enzymes'. Enzymes are powerful and highly specific catalysts.

Proteins, whether enzyme or otherwise, are the key intermediate stage by which information held in DNA specifies the functioning of the cell. Chemical processes studied in Molecular Biology either involve proteins directly or have proteins catalysing the reactions.

In regions of DNA that code for protein a run of three consecutive bases is used to specify selection of one amino acid. Three bases gives 64 possibilities rather than twenty. The encoding scheme has redundancy in it; several possibilities usually encode the same amino acid.

DNA is not translated directly. Instead a ribonucleic acid (RNA) copy of the DNA sequence is made, a process called 'transcription'. RNA is very similar to a single strand of DNA except uracil, a base like thymine but lacking a methyl group, is used in place of thymine and the sugar is ribose instead of deoxyribose. Instructions in an RNA transcript are read as a protein is formed in a process called 'translation'. This process of precisely controlled polymerisation takes place in complex assemblies, the ribosomes, which are themselves made from protein and RNA molecules.



1st base \ 2nd base	u	u	c	c	g	g	a	a
3rd base	u	c	u	c	u	c	u	c
u	F	F	S	S	C	C	Y	Y
3rd base	g	a	g	a	g	a	g	a
u	L	L	S	S	W	*	*	*
c	L	L	P	P	R	R	H	H
c	L	L	P	P	R	R	Q	Q
g	V	V	A	A	G	G	D	D
g	V	V	A	A	G	G	E	E
a	I	I	T	T	S	S	N	N
a	M	I	T	T	R	R	K	K

*Figure 1.5: The coding scheme whereby triples of adjacent bases (codons) code for individual amino acids. *'s indicate stop codons, the end of a translated section. The code is presented in the alphabet of RNA in which u substitutes for t. To produce proteins, a short transcript of RNA is first produced from the DNA master copy. The residues shown shaded are those encoded by three or more different codons. The organisation and shading of this table are non-standard and draw attention to a pattern in the code which involves codons whose second base is a.*

The diversity in proteins is achieved with great economy. The same molecular machinery driven from different sets of instructions is used in synthesising all proteins. It is variation in the sequences of the amino acid subunits, rather than a wide range to the subunits themselves, that give proteins their profoundly different properties.

Software for sequence analysis

Software for sequences analysis can be used to compare any newly determined protein or DNA sequence to sequences in the databases. This is a vital step in relating new information to information that is already known. When an unexpected new similarity is found between sequences, it can lead to a hypothesis about the processes taking place in cells. The hypothesis can then be tested by experiment. As an example, one similarity discovered by a computer database search linked proteins that stimulate cell growth (growth factors) and proteins implicated as causative agents in cancer (oncogenes) (Doolittle *et al.*, 1983). A number of different examples of growth factor and oncogene similarities are now known. The discovery of this link has been of great interest to researchers as it gives insight into the molecular mechanism whereby oncogenes lead to uncontrolled growth (Bradshaw, 1987).

Computers are of importance in other aspects of sequence analysis work in addition to their role in database searching. One of the most widely used software packages for biological sequence analysis, at least in universities, is the genetics computer group (GCG) package originally written at the university of Wisconsin (Devereux *et al.*, 1984). The GCG package provides a wide range of sequence analysis facilities from simple programs that reverse and complement a DNA sequence to derive the sequence of one DNA strand from its complementary pair, to computationally demanding programs that attempt to predict some structural aspects of RNA molecules. The distributors of the software have a policy of making source code for all these programs available. This makes it possible to adapt GCG programs to test out new sequence analysis ideas. The package provides a natural base from which to develop new ideas for sequence analysis.

Applied information technology

How can one develop new algorithms that will actually help molecular biologists? It is important that algorithms are genuinely of use, rather than solving problems so idealised that they bear little relation to biologists' requirements. Three approaches are considered.

1) Firstly, one may be able to identify aspects of sequence analysis which have been consistently neglected by software developers. One area that has been neglected is the combined use of both DNA and protein information.

Most analysis software deals independently with DNA data or with protein data, except when translating to or from DNA. Analysis programs therefore encourage researchers to see DNA and protein properties as independent, not to look for relationships between them. There is, however, interest in analyses that relate the two kinds of data.

One hypothesis where examination of both DNA and protein sequences is important concerns introns and protein domains. Introns are stretches of nucleic acid sequence which are removed from RNA before translation. Protein domains are independently folding functional regions within a protein. A hypothesis that links introns and boundaries between protein domains (Gilbert, 1978) is one of several that attempt to explain the presence of introns. Under this hypothesis introns separate functional domains and facilitate their rearrangement to make multifunctional proteins. Such a mechanism may underlie patterns of similarity actually observed in protein molecules (Patthy, 1985).

A second example where an interplay between DNA and protein properties is important concerns the use of rare codons. Some codons for the same amino acid residues are used preferentially to others. Use of the rarer codons causes pauses in translation (Varenne *et al.*, 1984) which may facilitate protein folding. Whilst codon usage for whole DNA sequences can be tabulated, software tools, as they currently stand, do not encourage investigation of such hypotheses.

Those two examples are somewhat specialised. However, there is a frequently required analysis in which consideration of both DNA and protein sequences is important. Currently in searching databases researchers use either DNA sequences or use the derived protein sequences. The problems this causes are considered in Chapter 9 where software that simultaneously uses both translated and untranslated DNA is presented.

2) A second approach to applying Information Technology is to examine existing computer sequence analysis tools that have proved their worth and to improve them. This may involve making a program run faster, making it perform a more comprehensive analysis or provision of a new interface that makes a cumbersome investigation more straightforward.

3) A third approach is to start from specific analysis problems that molecular biologists find are difficult to solve using existing tools. Either a satisfactory way can be found using a combination of existing tools, or new methods can be developed.

All three approaches have their uses. The approaches reinforce each other since new methods, improvements and difficult analyses influence the design of new software. It was the combined use of these approaches, new ideas being tested by writing new software, that directed research in this thesis.

In the early stages of the work a number of ideas and test implementations of programs were tried. Also time was spent helping biologists new to the GCG package with specific analysis problems. Some individual software ideas and conclusions from this work are summarised in Appendix 1. This summary illustrates some of the current practical difficulties with existing sequence analysis software. These early investigations helped to focus further research by drawing attention to the importance of sequence comparison in sequence analysis work.

Importance of sequence comparison

Comparison of biological sequences is fundamental to Computational Molecular Biology. It is possibly the most useful computational technique available. Why should this be so?

Firstly, comparison is required in database searches. Comparison allows new sequence data to be related to previously studied sequences. Similarity in sequences may give a researcher insight into a previously unsuspected function. Comparison of a sequence to sequences in a database allows the researcher to draw on a large collection of information that is frequently being updated.

Secondly, comparison of sequences which are already known to be related shows patterns of similarity and differences. Most regions of related sequences show some changes. Some regions show a remarkable stability. Sequence comparison can draw attention to these conserved regions. One can hypothesise that in these regions the evolutionary process has eliminated organisms which show variation. If so, this suggests that the regions have crucial biochemical functions requiring precise spatial organisations of amino acid residues. Comparison which reveals conserved regions can therefore act as a guide to identification of the site where an enzyme interacts directly with a substrate, the active site. This can guide an experimenter, increasing the chance of identifying by experimental techniques crucial regions early in their study.

Comparison software is also used to organise experimental data. In experimental work to determine a DNA sequence many fragmentary sequences are collected. Overlaps between these need to be found so that a long consensus sequence can be determined. Organising the fragments into a longer sequence requires comparison.

Underlying the various applications of sequence comparison is a common theme. Comparison is a first stage in organising information. In biological sequence database searching, comparison selects and brings to the attention of researchers information that is most likely to be relevant. The comparisons bring related sequences together whatever their order in the database. In studies of related sequences, comparisons organise the differences and similarities so that patterns of similarity can be seen.

Alternatives to sequence comparison

The three dimensional structure of a protein, as for example deduced from X-ray crystallographic data, gives information about the arrangement in three dimensions of the atoms of the molecule. In contrast, chemical structures only give information about covalent bonding. Chemical structures give selective information about the distances between atoms. Where the word 'structure' is not qualified by 'chemical' in this thesis, we are referring to three dimensional structure.

Comparison of protein structures should give a more accurate method for investigating shared function than does sequence comparison alone. Function may depend on the precise arrangement of residues in a small patch at the surface of the protein. Residues which are close in the folded protein will not necessarily be close in the linear sequence.

However, structural comparison techniques have limited applicability. Only a few hundred distinct protein structures are known whereas many thousands of protein sequences have been obtained (Pallabiraman *et al.*, 1990). This reflects the difficulty of obtaining protein structures. Determination of a structure using X-ray crystallographic techniques is only possible where the protein can be crystallised. The newer and more rapid nuclear magnetic resonance (NMR) structure determining techniques also have limitations, particularly as regards the size of molecules that can be analysed (Gronenborn & Clore, 1989).

Were it possible to deduce protein structure directly from sequence, sequence comparison would have far less importance. Structural rather than sequence comparisons would be of greater interest to researchers attempting to understand protein function. Attempts have therefore been made to apply computers to the problem of deducing protein structures from protein sequences.

Predicting structure from sequence

Protein structure prediction from sequence data alone presents a formidable problem. Each structure for a protein has a corresponding energy. The energy depends to a large extent on hydrogen bond formation. Calculating the structure of minimum energy for a protein of known chemical structure involves minimisation of a non-linear energy function involving thousands of variables. Approaches to the problem so far have met with very limited success. Simulations of protein folding and use of current energy minimisation techniques suffer from phenomenal computational demands. There is uncertainty too about whether the structure a protein adopts in a cell corresponds to the structure with minimum energy, even were it possible to perform the minimisations in reasonable times (Zvelebil *et al.*, 1987).

Dynamic protein simulations can currently handle minor disturbances of known structures and timescales of the order of picoseconds. Protein folding by contrast involves major structural changes and can take seconds (Hantgan *et al.*, 1974). The conformation of a protein, moreover, may depend on the presence of other cellular components such as other proteins that catalyse the folding process (Sambrook & Gething, 1989).

An alternative to simulation and energy minimisation techniques is to use statistical properties of sequences. Attempts have been made to employ statistical relationships between short sequences and common structural motifs to predict the presence of alpha helix and beta sheets. The poor success of these statistical methods has generally been ascribed to 'tertiary structure effects', that is, interactions between residues distant in the linear sequence (Kabsch & Sanders, 1983).

Without structural prediction, structural comparison is limited to those sequences whose structure has been determined experimentally. On the other hand, sequence comparison can be applied to all proteins in the sequence databases. By comparing sequences rather than structures researchers are potentially able to relate their sequence to a far larger class of proteins and they are able to do so long before a structure for their sequence is available. Such comparisons can even be of help in determining protein structures. Some of the preliminary structural models that researchers work with are based on sequence similarities of the protein being modeled to proteins of known structure (Ripka, 1986).

Concluding remarks

Sequence comparison techniques are needed in many areas of Computational Molecular Biology. They make possible automatic organisation of sequence data to draw attention to biologically significant patterns. The applications range from the early stages of determining a sequence to advanced studies of the mechanism of protein action. Accordingly sequence comparison techniques and associated software play a central role in this thesis.

Chapter 2: Comparison Methods and the NWS Algorithms

This chapter examines the methods involved in measuring sequence similarity. Computers can easily find strong similarities between sequences such as runs of twenty or more identical residues. Comparison should draw attention to weaker similarities as well as to the very strongest similarities. Weaker similarities must be picked up from a background of similarities that occur by chance and which do not reflect biologically significant relationships. In detecting the weaker matches the crucial property of comparison algorithms is their ability to discriminate between biologically significant matches, 'signal', and chance matches, 'noise'.

To detect biologically significant similarities, methods are needed for converting a qualitative property, the similarity of two sequences as judged by the biologist, into a quantitative measure that can be calculated by the computer.

Programs for finding similarities are, in effect, algorithmic descriptions that approximate to biologists' intuitions about what signal matches are like. An algorithmic description can readily capture some aspects of sequence similarity that distinguish genuine sequence relationships from spurious ones. For example, in two related proteins there is a good chance of finding many short runs of two or three residues that are present in both sequences. This kind of similarity is rarer in unrelated sequence pairs. The simplest algorithms for measuring relatedness are 'word based' algorithms that rely on such runs. Modifications to these methods can improve detection of biologically interesting similarities. This is described in the following sections. These lead to a description of comparison 'by alignment' and to description of the Needleman Wunsch Sellers (NWS) algorithms for finding optimally scoring alignments.

Word based comparison

'Words' are contiguous sequences of characters within a longer sequence.

At its simplest a word based comparison method would count the number of words of a fixed length shared by two sequences. For example the two sequences:

```
Seq1:  FLTFERNRQIC
Seq2:  FLSDKNRYQIC
```

have four two letter words in common. These words are FL, NR, QI and IC.

One attraction of word based comparison methods is that algorithms for counting matching words can be extremely rapid. Words contained in both sequences can be located very efficiently using standard sorting and indexing algorithms (Knuth, 1973a).

Estimating likelihoods of chance matching

With word based methods a simple model gives some idea of the expected level of chance matching. Were all amino acids equally abundant in proteins, two words of length six chosen from two sequences of random amino acid residues would have a probability of $1/20^6$ (which is 1.56×10^{-8}) of matching identically.

Not all amino acids are equally abundant. The probability of the first residue of two random words both being glycine, using the amino acid frequencies of figure 1.2, is $0.089^2 = 0.0079$. The probability of both being tryptophan is $0.01^2 = 0.001$. Summing these probabilities over all amino acids gives the probability of an identical match which is 0.07, i.e. a 1 in 15 chance of matching. Two random words of length six from proteins of average composition have a probability of $1/15^6 = 8.78 \times 10^{-8}$ of matching by chance.

There are about 90,000 ways of choosing two six letter words from two sequences of length 300. The expected number of matching words of length six in two sequences of length 300 is $90{,}000 \times 8.78 \times 10^{-8} = 0.008$. Finding such a word would tend to indicate that the sequences were related.

Such figures provides only a useful rule of thumb. Treating proteins as random sequences of amino acids does not accurately reflect patterns present due to biological constraints. A protein sequence may have local regions of biased composition (McQuay, 1991). It may for example contain a region rich in hydrophobic residues located in the protein core. Other proteins which do not have similar functions may contain a similarly biased region, increasing the chance of a matching word above the normal noise level. In practice this problem is dealt with by the biologist interpreting the computer's results, rather than by the computer. This is one reason why it is important that comparisons give not only quantitative scores but also show the regions of sequence similarity.

Further problems arise when trying to calculate the likelihood of matching when comparing one sequence to sequences in a database. Protein databases contain families of related proteins. A similarity to one member of a family implies a high likelihood of similarity to other members. Comparing a sequence of unknown function to all sequences in a database and finding ten sequences that show evidence of similarity to it may not be much more surprising than finding just one, if the ten sequences are closely related to each other. In a model for random matching it is simpler, though not accurate, to treat the proteins in the database as unrelated to each other.

These comments serve to illustrate that measuring likelihoods of chance matching is problematic even with the simplest of similarity measures. The model for random matching necessarily makes simplifying assumptions. The likelihood measures can, however, give guidance at extremes of similarity. They can suggest that a similarity is so strong that some biological explanation for it is required, or that a weak similarity is so poor that its occurrence can be entirely explained as chance matching.

Ultimately the test of a method for scoring similarity is whether or not it leads to new insights into protein function validated by actual experiment. Rather than significance by an arbitrary numerical measure, it is significance to the biologist that matters.

Substitutions

The requirement that words match exactly makes methods based on exact word matching liable to miss similarities that are of significance to biologists. Information about amino acid similarity can be used to improve the sensitivity of a protein sequence comparison method. To the biologist chemical similarities which suggest similarities of function are of interest. The chart of amino acid sidegroups in figure 1.3 draws attention to some chemical similarities between amino acids. Serine and threonine, for example, have sidechains of ethanol and propanol. These two alcohol sidechains differ in length by only one carbon atom. Serine can and does substitute for threonine in many related proteins. Exact matching of words would fail to identify two words which differ only by an S replacing a T. Such inexact matching would be of interest to a biologist.

Alphabet reduction

Insensitivity to inexact matching can be alleviated by 'alphabet reduction'. Alphabet reduction represents the protein sequences using a more restricted alphabet. The reduced sequences are the same length as the originals but some characters are replaced by characters representing related amino acids. For example, all occurrences of T could be replaced by S. Exact matching of the alphabet reduced words corresponds to inexact matching of the actual sequences. Alphabet reducing the example from page 18 using S for T, D for E, K for R and F for Y, all of which are amino acid residue pairs with similarity, we get:

```
Seq1: FLSFDKNKQIC
Seq2: FLSDKNKFQIC
```

A pair of sequences which now have seven words of length two in common.

Evidently the alphabet reduction process can only be taken so far. With too much alphabet reduction it becomes impossible to distinguish proteins which are related to each other from those which are not. It has been shown that alphabet reduction gives an improvement to sensitivity of comparison only when reducing the pairs (D,E), (F,Y), (K,R), (I,V) and (L,M) (Collins & Coulson, 1987).

Scoring a word

Even with alphabet reduction a single character pair mismatch can prevent two otherwise similar words from being matched. Matching below a certain level is then not detected at all. Two closely related sequences could have many words which are nearly the same in common. Matching of alphabet reduced words could fail to make use of much of the evidence for relatedness. This is particularly problematic if longer words are used since longer words are less likely to match exactly than shorter ones. This problem limits the ability of word matching to find weaker signals.

The problem of a few changes preventing otherwise similar words being detected can be overcome. Words pairs can be scored by counting the number of character similarities either with or without alphabet reduction. This makes possible the detection of words with above average matching even when the matching is imperfect.

Scoring amino acid similarity

Once the similarity of words is scored rather than simply being 'present' or 'absent' it is only natural to do the same with the individual amino acid similarities. This approach leads to scoring that discriminates better between signal and noise than counting similarities after alphabet reduction does.

Similarity between pairs of amino acids is scored by use of a table of values. In the table S has its highest score against S, scores less highly against T and scores negatively against most other amino acid residues. Exact matching is rewarded more strongly than inexact matching in contrast to the case with alphabet reduction. Using an amino acid scoring table in scoring words, scores can better reflect the evidence for relatedness. Matching of rare amino acids, for example, tends to give greater evidence for a genuine relationship than does the matching of commonly occurring residues. This is reflected in high scores for matching W against W and C against C, tryptophan and cysteine being two of the rarest amino acids. Derivation of sensitive amino acid similarity scoring tables for detecting evolutionary relationships is discussed in Chapter 4.

An amino acid scoring table that scores one for each exact match of amino acids and zero for mismatches can be used to give a score that counts exact matches. Tables consisting of ones and zeroes can also be constructed to count matches after alphabet reduction. An algorithm that scores using an amino acid scoring table can thus readily be used to score for exact matching or for matching after alphabet reduction.

Locating high scoring word pairs

Forsaking exact matching of complete words and scoring word similarity instead has a major disadvantage. The fast sorting methods that made the word based methods so attractive can no longer be exploited. If words differ in their first letter they are likely to be far apart after sorting yet if other letters agree their similarity scores can be high.

The most straightforward algorithms to locate high scoring word pairs take each word from one protein and compare these in turn with every word in the other protein. Fortunately more rapid algorithms exist. These use the first comparison of a word to limit the subsequent choice of words to compare against, narrowing the search using a so called 'Post-office tree' (Knuth 1973b). The word comparisons use some 'metric' for the similarity of words. Scores must measure the difference between words rather than the similarity. Conversion to a metric measure can easily be made in the case of scoring exact matches after alphabet reduction. For scoring with alphabet reduction, counting mismatches rather than matches after alphabet reduction gives a metric difference measure. Once similar words are found the difference scores can be converted to similarity scores if desired.

These fast word comparison algorithms which find word similarity rather than word identity have apparently not been used in biological sequence comparison. They are not used in this work either. There is a fundamental problem that has not yet been mentioned with any method that is based on word matching. Missing or extra residues within the words are not accommodated. There are, however, well tried algorithms that deal with both inexact matching and missing or extra residues in either sequence.

The methods which accommodate insertions and omissions are popular amongst biologists as the comparisons they make are sensitive to the kind of changes biologists expect to see in related proteins. A disadvantage of these methods is that they are computationally expensive. Consequently they are used for detailed comparison of sequence pairs which are already known to be related, database searching being most usually performed using word based methods.

Comparison by alignment

The sensitive algorithms compare sequences 'by alignment'. An alignment of two protein sequences presents the sequences in a manner which draws attention to similarities between them. A pairwise alignment of proteins shows two similar protein sequences one placed above the other. An alignment is shown below:

```
      **    ** ***
Seq1: FLTFERNR-QIC
Seq2: FLS-DKNRYQIC
```

Gaps, represented by '-', have been inserted in the upper and lower sequences to improve the matching in each column. In addition a '*' has been placed above each identical residue pair.

Every alignment of two sequences has an associated score. Each pair of aligned residues contributes to the score. Similar or identical residues in the same column of the alignment contribute positively to the score. Aligned dissimilar residues reduce the score. As before, the amino acid similarity scores come from a table. Gaps in the alignment in either sequence represent insertions or deletions relative to one or other of the sequences and are called 'indels'. Each gap reduces the alignment score by an amount referred to as an 'indel penalty'.

The algorithms for scoring similarity find the highest scoring alignment of the two sequences. High indel penalties encourage alignments with few gaps whereas more lenient penalties are conducive to gaps.

Having described what optimal scoring alignments are, we now describe an algorithm which finds them.

The local homology algorithm

The alignment algorithm described here is a particular variant that finds 'local regions of homology'. It is known as the Type III algorithm being one of a family of related algorithms; the Needleman Wunsch Sellers (NWS) comparison algorithms (Needleman & Wunsch 1970). The Type III variant is due to Smith and Waterman (1981). Variants of the algorithms have uses in diverse comparison applications such as speech recogniton, error correction of formal languages, analysis of birdsong and RNA structure prediction (Kruskal, 1983).

Enumerating all possible alignments and calculating their scores is too computationally expensive to be practical. Even for short sequences the number of possible alignments is large. The number grows exponentially with sequence length. Nevertheless the problem of determining which of these has highest score can be solved in time proportional to the product of the sequences' lengths. Dynamic programming techniques are used. In general dynamic programming techniques work by a systematic decomposition of the problem into simpler ones (Sedgewick, 1983). Larger sub-solutions are built up from smaller ones. For this problem high scoring alignments are built on optimal initial portions.

Decomposition of the problem rests on the following observation: the initial portion of any optimal alignment must itself be an optimal alignment of two shorter sequences.

Characteristic to dynamic programming is the regularity of decomposition. In this problem the sub-computations can be organised on a rectangular array called the 'match matrix' (sometimes also 'path matrix'). There is then a correspondence between an alignment of two subsequences and a path in the matrix. The top edge and left edge of this matrix correspond to the two sequences being compared. Diagonal steps in the matrix represent matches of two amino acids. Horizontal steps correspond to placing a gap against a residue in the first sequence. Vertical steps correspond to a gap against a residue in the second sequence. Figure 2.1 show a path made up from such steps. Horizontal and vertical steps incur an indel penalty whereas diagonal steps score for the residue pair similarity, which in general may be positive or negative.

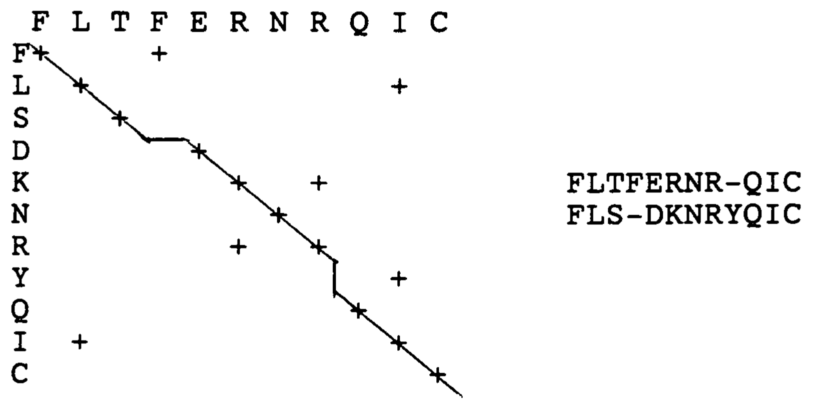


Figure 2.1: Correspondence between a path in the match matrix and an alignment. '+' signs indicate positively scoring steps. Each cell ends up holding the score for the best path ending in that cell. These scores are not shown.

When calculation of the entries in the match matrix is complete, each cell holds the score for the best path that ends at that cell. The score is also the score for the best initial portion of an alignment that ends at a certain position in each of the two sequences. The score in each cell depends on the scores in three neighbouring cells, the cell above, the cell to the left and the cell diagonally up and to the left. The best path ending at a cell is either an extension of a best path ending in one of the three neighbouring cells or is a new path which starts and ends at this cell. If a new path is started the score is reset to zero. The score of an extended path is the score of the cell it starts from adjusted by the indel penalty, for horizontal and vertical steps, or by the score for aligning two residues if the extension is a diagonal step. The score placed in a cell is the highest of the scores of the four possible paths ending in it, see figure 2.2. Cells along the top edge and first column treat non-existing neighbour cells as if they contain zero scores.

The maximum score in the entire matrix gives the score for the optimal sequence alignment of the two sequences being compared. The path ending at the cell with the maximum score corresponds to the optimal alignment.

$$\begin{tikzcd}
90 \arrow[dr, "+3"] & 91 \arrow[d, "-10"] \\
71 \arrow[r, "-10"] & 93
\end{tikzcd}$$

Figure 2.2: The score for a cell is the score of the best extension of the paths ending in three neighbouring cells or zero if all three of these have negative score.

Each cell computation requires a small fixed number of arithmetic operations. Since the number of cells is the product of the sequence lengths, computing all cell values has time complexity $O(n^2)$ where n is the length of each sequence (assumed equal). This *'O'* notation gives an asymptotic measure of performance (Knuth, 1981). Time complexity *'$O(n^2)$'* means that the execution time for the algorithm divided by n^2 approaches a constant value for large n. For sufficiently large n an algorithm with $O(n)$ time complexity will be faster than an $O(n^2)$ algorithm. In contrast to the alignment algorithm described here, the rapid exact matching methods have time complexity $O(n \, Log \, n)$, provided suitable assumptions are made about the word size used. A sufficient condition is that the word size be proportional to $Log \, n$. For fixed word size, location of exactly matching words is $O(n^2)$ too.

The speed of an algorithm can also be reported as the number of pairwise sequence comparison performed in a fixed time. To make this independent of the sequence length, multiplication by the product of the lengths gives the number of path matrix elements (PMEs) computed in unit time.

Reconstruction of alignment

The procedure described finds the maximum score of an alignment. An algorithm is also needed to trace the correct path through the match matrix and reconstruct the alignment. Reconstruction of the alignment is a more rapid process than computation of the comparison score. It is an $O(n)$ process provided the scores in the match matrix have already been computed and stored and the highest score located.

To perform the reconstruction the highest scoring cell is designated the current cell. Starting at this cell individual steps to the left, up, or diagonally up and to the left are taken. At each cell there is a choice between three possible steps; that is steps against the direction of the arrows in figure 2.2 . One of these steps must lead to a cell with a score high enough to produce the score at the current cell. This cell becomes the new current cell. Each of the steps taken is on the maximum scoring path. The process of taking steps in reverse is repeated, changing the score with each step, until the score reaches zero. All steps in the alignment have then been reconstructed. Although the steps are generated in reverse order this is easily corrected before an alignment is presented.

Sometimes equally good alternatives for some parts of the path exist. In these cases, which of the possibilities is chosen is dependent on details of how the algorithm is programmed.

Pointers in reconstruction

Usually a variant of the path reconstruction algorithm is described which uses stored information about which path enters each cell. This information is recorded at the time the score for a cell is calculated and takes the form of a pointer to the cell whose path was extended. Path reconstruction then simply follows these pointers. Keeping pointers as well as scores increases the time and storage requirements of the computationally most expensive part of the alignment procedure, the $O(n^2)$ part. Pointers are not required when using the method for reconstruction described in the previous section which uses only the scores.

Types of alignment

There are three main variants of the alignment algorithm. These are designated by different numbers (Lyall *et al.*, 1986).

Type I	Best complete sequence alignment.
Type II	Best location of one sequence within another.
Type III	Best local homology.

The Type III version finds the best local similarity between two sequences. It finds a similar region contained in both sequences.

If negative scores are not reset to zero and new paths started, then the algorithm finds the best alignment of complete sequences. This is the Type I variant. It produces an end to end alignment including all residues of both sequences.

An intermediate variant, the Type II, finds the best location of one sequence within another. This might be suitable for looking for complete motifs within a protein. Motifs are short patterns that have a well characterised function, one such being the ATP binding motif. The Type II variant resets scores to zero only for the top edge of the matrix and finds the best path ending in one of the cells at the bottom edge of the matrix. This corresponds to alignment of the entire motif with an absence of penalties for unaligned residues before and after the motif.

Each of the three methods has its uses. The Type III is most appropriate for database searches where the extent of any region of similarity is not known in advance and cannot be presumed to include the whole of any sequence. Type II and Type I effectively force the entirety of one or both sequences, respectively, to be included. Type III is capable of aligning whole sequences if the similarity extends along the whole length of the sequences.

A major disadvantage of these three algorithms is their computational demands. Their major advantage is their ability to detect similarities of a kind that are of interest to biologists that exact matching word based methods described earlier cannot find. This sensitivity arises because comparisons by alignment can find matching words that are interrupted by insertions and deletions. Also, with alignment based algorithms, the individual residue matches are scored using values from discriminating amino acid similarity scoring tables.

Chapter 3: Measuring Similarity

The sensitivity with which comparison between sequences can be made is dependent on the similarity scoring scheme used for scoring amino acid pairs. In this chapter we look at how to measure amino acid similarity in a manner that is suitable for detecting evolutionary relationships. We are therefore interested in the extent to which pairs of proteins have diverged from a common ancestor.

Divergence measures

One of the simplest assessments of how far a pair of related proteins have diverged uses an alignment of the sequences. The percentage of aligned residues which differ gives a convenient divergence measure. Identical sequences will be 0% divergent by this measure. For two unrelated sequences there will be a certain level of chance matching. When comparing unrelated protein sequences with the amino acid composition given in figure 1.2 there is 7% amino acid matching just by chance and thus 93% divergence by the measure.

A measure of divergence which is closer in spirit to measuring an evolutionary distance is due to Dayhoff *et al.* (1978). They developed a unit of measure called 'The PAM'. This gives the number of accepted mutational events per hundred residues. The emphasis on 'accepted' is to distinguish changes in proteins which lead to viable organisms, i.e. changes which evolution accepts, from those which do not and which are not normally observed. Accepted changes are changes that are in the proteins of organisms which survive. In principle the underlying mutational changes could be very different from these. In practice we are only interested in the mutations in observed proteins and treat mutations accepted by evolution as the only sort which occur.

The Dayhoff model

The measure PAMs is integral to a model that predicts amino acid substitution frequencies between residue types. For each evolutionary distance measured in PAMs the model gives a table of frequencies for substitution between each pair of residue types, including entries for the frequencies of residue types remaining unchanged. Each table therefore implies a certain level of amino acid identity in two proteins related at that evolutionary distance. There is thus a correspondence between PAMs and percentage amino acid difference. The graph in figure 3.1 shows this correspondence over a range of values. The number of PAMs is invariably higher than the percentage difference. This arises because multiple mutations at the same site may reverse previous changes. In fact 100 PAMs, that is 100 changes per 100 residues, corresponds to an observed amino acid difference of 57%. At 100 PAMs we still expect to see substantial evidence for evolutionary relationship. As PAMs increase the percentage amino acid difference asymptotically approaches 93% corresponding to protein sequences that show matching only by chance.

For small evolutionary distances the frequency of multiple mutations at the same site is low. Here divergences measured in PAMs and in percentage differences agree quite closely. 15 PAMs for example corresponds to 13.6% amino acid difference.

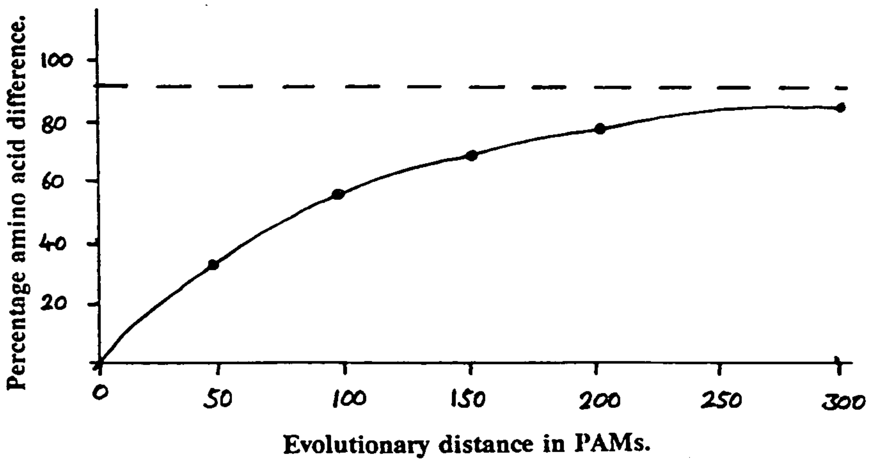


Figure 3.1: Correspondence between PAMs and percentage amino acid difference. After Dayhoff et al. *(1978).*

Use of the amino acid substitution frequencies.

The main use of the amino acid substitution frequencies is to derive tables for scoring amino acid replacements for various evolutionary distances. Such tables have a score for each possible pair of amino acids. These scores are used to measure evidence for sequence relatedness. The scores combine information from two frequency tables. One table gives the expected frequencies of replacements of each residue type by each residue type, at a particular evolutionary distance in PAMs. This table gives substitution frequencies for signal matches at the chosen evolutionary distance. The other table is the limiting case for infinite PAMs. It gives the frequencies of residue types being paired in random alignments of unrelated proteins. This table gives expected substitution frequencies in noise matches. These two frequency tables are combined to make score tables which measure how much more likely two aligned residues are to be from related rather than unrelated sequences. The scoring tables are used to assess the quality of an alignment of two proteins and are important in automatic methods for alignment. The scoring tables are referred to as 'PAM tables'.

Assumptions of the model.

The Dayhoff model is based on observed frequencies of changes in aligned pairs of closely related proteins. These frequencies of change are extrapolated to predict substitution frequencies in more distantly related proteins. The model makes a number of simplifications to make the extrapolation. In the model the probability of change to a particular new amino acid at a particular site depends only on the amino acid currently there. These probabilities are 'transition probabilities'. Implicit in this simplification are three assumptions that are of questionable biological accuracy. These are not clearly stated in the original paper and so are set out here.

- Independence of sites.
- Equivalence of sites.
- Temporal independence.

The first states that changes at any one site are not influenced by nor correlated with changes at another. The second is that all sites with the same amino acid behave in the same fashion. The third is that the transition probabilities do not change with time. The process of change is assumed to be uniform. For example, the model does not consider sudden changes of a different kind to the gradual changes observed in closely related proteins.

Equivalence of sites is known to be inaccurate. A paper by Perutz and Lehmann (1968) describes many examples of changes between residues in naturally occurring mutant haemoglobins. Of these, two examples concern changes between leucine and arginine. At residue 14, interchanges between these residues do not lead to any clinical symptoms. At residue 92, leucine is normal and arginine leads to polycythaemia. Evidence that different sites with the same residue have different mutational properties is also provided by multiple sequence alignments of functionally equivalent proteins (Chapter 6). The range of variation at different sites varies enormously. Some sites require specific residues whereas others can tolerate variation.

Independence of sites is also open to question. The frequencies of occurrence of residues are known to be influenced by the neighbouring residues (Claverie & Bougueleret, 1986).

In scoring amino acid similarity, sites are treated as independent and equivalent. These two simplifications are implicit in any scoring scheme that scores pairs of amino acid types. Thus one uses some kind of average behaviour in producing the score. This does not mean that taking average behaviour is a necessary or desirable part of the extrapolation process. Extrapolating averaged changes will not in general give the same answer as extrapolating changes and then averaging. In theory at least, a model that allowed for differing evolutionary constraints at sites containing the same amino acid could yield superior scoring tables. This is clearly shown by considering a protein that has half of its sites absolutely fixed. An extrapolated model based on average behaviour of sites would show that nearly all sites change at sufficiently large evolutionary distance. Extrapolating and then averaging would show that on average just over half the sites stayed fixed.

The range of different constraints on sites is unknown. There is probably insufficient sequence data to characterise the constraints to a level where extrapolating changes at different kinds of site with the same residue would be feasible. A possible exception to this is at sites containing cysteine. For cysteines additional information about disulphide bonding is sometimes available. This could be used to distinguish two cases, cysteines involved in disulphide bonding and those not. A case could be made for extrapolating these two cases as if the cysteines were two different residue types, cystine (participating in a disulphide bond) and cysteine (not involved in a disulphide bond).

The Dayhoff model has also been criticised from other viewpoints. Wilbur (1983) perhaps misunderstood the notion of accepted mutation. Dayhoff's model is based only on changes at the amino acid level which are accepted by the evolutionary process. Underlying mutational changes in DNA are only indirectly reflected. Wilbur's criticism of the model is that it is inconsistent with an alternative model based on underlying independent changes at the DNA level, all of which are accepted.

In spite of the model's shortcomings, real and imagined, the tables it produces are the basis for the most sensitive methods for comparing pairs of protein sequences. Dayhoff was able to show that other tables based on identities only and the genetic code had significantly poorer discrimination between related and unrelated proteins (Schwartz & Dayhoff, 1978). Ultimately the discriminating ability of the scoring schemes the model leads to is the justification for using the Dayhoff model.

A modified presentation of the Dayhoff derivation is now given. This clarifies the link to the mathematics of Markov processes (see e.g. Revuz, 1975) and makes explicit the two parts of the model, one part to model signal, the other noise. This also gives an opportunity to clarify some questions raised by the original exposition and to introduce notation which is needed in the discussion of discriminatory power.

Dayhoff's original paper is frequently found somewhat opaque. A diagrammatic overview of the stages of the derivation may help.

	C	S	T	P	A	G	N	D	E	Q	H	R	K	M	I	L	V	F	Y	W	B	X	Z
C	12																						
S	0	2																					
T	-2	1	3																				
P	-3	1	0	6																			
A	-2	1	1	1	2																		
G	-3	1	0	-1	1	5																	
N	-4	1	0	-1	0	0	2																
D	-5	0	0	-1	0	1	2	4															
E	-5	0	0	-1	0	0	1	3	4														
Q	-5	-1	-1	0	0	-1	1	2	2	4													
H	-3	-1	-1	0	-1	-2	2	1	1	3	6												
R	-4	0	-1	0	-2	-3	0	-1	-1	1	2	6											
K	-5	0	0	-1	-1	-2	1	0	0	1	0	3	5										
M	-5	-2	-1	-2	-1	-3	-2	-3	-2	-1	-2	0	0	6									
I	-2	-1	0	-2	-1	-3	-2	-2	-2	-2	-2	-2	-2	2	5								
L	-6	-3	-2	-3	-2	-4	-3	-4	-3	-2	-2	-3	-3	4	2	6							
V	-2	-1	0	-1	0	-1	-2	-2	-2	-2	-2	-2	-2	2	4	2	4						
F	-4	-3	-3	-5	-4	-5	-4	-6	-5	-5	-2	-4	-5	0	1	2	-1	9					
Y	0	-3	-3	-5	-3	-5	-2	-4	-4	-4	0	-4	-4	-2	-1	-1	-2	7	10				
W	-8	-2	-5	-6	-6	-7	-4	-7	-7	-5	-3	2	-3	-4	-5	-2	-6	0	0	17			
B	-4	1	0	-1	0	1	2	4	4	2	2	0	1	-2	-2	-3	-2	-4	-2	-4	4		
X	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Z	-5	0	0	0	0	0	1	3	4	4	3	1	1	-1	-2	-2	-2	-5	-4	-5	4	0	4

Figure 3.1b: Dayhoff Matrix for 250 PAMs. In this figure only half the matrix is shown as the matrix is symmetrical about the diagonal.

The letters B X and Z represent ambiguous amino acid residues. B represents asparagine or aspartic acid (N or D), Z represents glutamine or glutamic acid (Q or E) and X represents any amino acid.

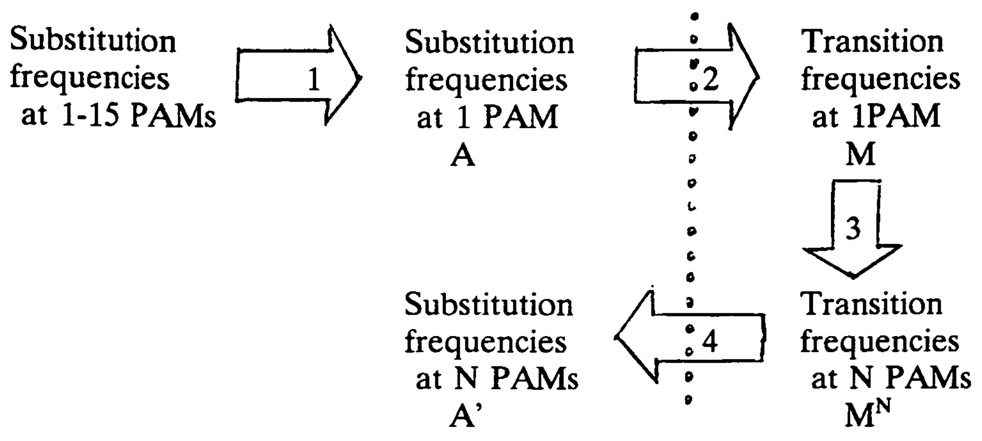

Substitution frequencies at 1-15 PAMs
1
Substitution frequencies at 1 PAM
A
2
Transition frequencies at 1PAM
M
3
Substitution frequencies at N PAMs
A'
4
Transition frequencies at N PAMs
M^N

Figure 3.1: Overview of the derivation of substitution frequencies at N PAMs in the Dayhoff model. Tables to the left of the dotted line are symmetric.

1 PAM substitution frequencies (Step 1)

An essential table that determines all others in the Dayhoff model is the residue substitution frequency table for sequences 1 PAM apart. This table, A, which is symmetric is derived from pairs of aligned sequences.

A_{ij} = Frequency with which residue i substitutes for j
$A_{ij} = A_{ji}$

In matrix notation the symmetry of A is expressed by:

$$A = A^T$$

When a sequence is aligned with another sequence which is 1 PAM divergent from it, we expect the substitution frequencies to be similar to those in A. Ideally the table A would be derived from observed frequencies of substitution in pairs of proteins that were at an evolutionary distance of 1 PAM. In practice the data is collected for proteins that are less closely related and a scale factor is used to reduce the level of substitutions to 1 per 100 residues and increase the fraction of unchanged residues.

A questionable practice used in collecting the initial data set was the use of inferred ancestral sequences derived form phylogenic trees to try to reduce the evolutionary distance between the pairs of sequences considered.

To derive a substitution frequency table from A for greater evolutionary distances we require a matrix giving probabilities of transitions from each amino acid to each other amino acid at 1 PAM. Dayhoff called this matrix the 'mutation probability matrix'. Here we use the term 'transition probability matrix' to emphasise the connection to the theory of Markov processes.

Transition probabilities (Step 2)

The columns of the 1 PAM transition probability matrix indicate how a protein consisting entirely of one amino acid type will change in a fixed evolutionary interval. The matrix is used to predict how a protein of arbitrary composition will change. The composition is represented as a vector. Pre-multiplying this vector by the transition probability matrix yields a new vector giving the new composition after an interval of 1 PAM.

One important composition vector gives frequencies of the amino acids in the sequences that produced the matrix A. This is taken as the average composition for proteins. The components f_j of the vector are given by:

$$f_j \quad = \Sigma_i A_{ij}$$

Using J to stand for the vector of all 1's this summation can be expressed in matrix notation by:

$$f \quad = A.J$$

Figure 1.2 in Chapter 1 lists values of the f_j's.

f, the average composition vector, is used in computing the transition probability matrix, M, for 1 PAM. M is obtained by dividing each column of A by the appropriate f_j. The twenty entries in each column of M give frequencies for the twenty possible transitions per unit occurrence of the amino acid represented by the column.

We can express this normalisation in matrix notation. We use diag($1/f_j$) to denote the matrix with diagonal entries $1/f_j$ and zeroes elsewhere. Then:

$$M = A.\mathrm{diag}(1/f_j)$$

One property of the matrix M was stated by Dayhoff without proof. When it acts on the composition vector f, the vector is left unchanged. This is proved below:

$$M.f = A.\mathrm{diag}(1/f_j).f = A.J = f$$

The preceding equation is exactly equivalent to the statement that f is an 'Eigen vector' for the matrix M with 'Eigen value' one. Markov theory shows that f is then the asymptotic composition; that is, acting repeatedly on any composition vector with M will produce a series of vectors converging to f.

Extrapolation to larger PAMs (Step 3)

In the model changes for large PAM distances are the result of successive changes occurring with frequencies represented in M. This is where the assumption of 'temporal independence' is used. Because of the associativity of matrix multiplication, M acting N times on a vector is the same as acting with M^N on that vector. The matrix of transition probabilities at N PAMs is then M^N. In the language of Markov processes this is the N stage transition probability matrix. From this matrix we wish to obtain a matrix giving substitution frequencies.

Substitution frequencies at N PAMs (Step 4)

In converting from a substitution frequency table to a transition probability matrix (step 2) the amino acid frequencies f_j were used. The f_j's are unchanged with transitions occurring at the frequencies in M and therefore also unchanged for changes occurring with the frequencies in M^N. Tables for substitutions for any PAM distance should have the same f_j's. Substitution frequencies are thus related to transition probabilities at the same PAM values by the f_j's. Multiplying the columns of M^N by the f_j's gives the N PAM expected substitution frequency table A'.

$$A' = M^N.\text{diag}(fj)$$

This gives the first frequency table from the model, the table for signal matching.

Random matching

Random matching frequencies are computed from the f_j's. The frequency of seeing residue i against residue j by chance is simply the product of frequencies f_i and f_j. This gives expected substitution frequencies if the two sequences were unrelated and of average composition, i.e. the frequency table for noise.

This then completes the derivation of the two frequency tables used in the Dayhoff model.

Score tables

The table A' giving the expected substitution frequencies at N PAMs can be used to see how closely a pair of aligned proteins fit the Dayhoff model. Marked deviation from the substitution frequencies predicted by the Dayhoff model would tend to undermine confidence in the alignment. In principle this could be used as the basis for an automatic method for producing good alignments. However, computational techniques for alignment require residue-pair scores.

To produce residue pair scores, both frequency tables are used. The scores measure evidence for relatedness under the model relative to the null hypothesis of random matching. The evidence for evolutionary relationship at N PAMs from an aligned pair of residues is the ratio of the frequency under the Dayhoff mutation model for related sequence matching, to the frequency assuming random matching. Where this ratio is greater than one it is a positive indication of relatedness. Where less than one, it is counterindicative.

For an alignment, these 'odds ratios' for each pair of residues can be multiplied together to give an aggregate value. To facilitate calculation the logarithm of each ratio is taken. Addition can then be used in place of multiplication. This log odds table for a particular evolutionary distance measured in PAMs is the scoring table suggested by Dayhoff. Positive values of the scores are indicative of relatedness, negative values counterindicative. It is usual to scale these scores up by a factor of 10.

$$X_{ij} = 10 \times \log(A'_{ij}/f_j f_j)$$

Integer values are found to be acceptable for calculations, which is important for rapid calculation. An important property of these scoring tables is their negative expectation for scoring amino acid replacements in unrelated protein sequence pairs. That is:

$$\Sigma f_i f_j X_{ij} < 0$$

We demonstrate this using the relationship *Log K* $\leq$ *K* - 1.

$$\Sigma f_i f_j X_{ij} = \Sigma f_i f_j \mathit{Log}\ (A'_{ij}/f_i f_j) < \Sigma f_i f_j\ (A'_{ij}/f_i f_j - 1) = \Sigma A'_{ij} - f_i f_j = 1 - 1 = 0$$

For Type III alignment this property is an essential condition since the condition for ending a path in the match matrix is that the score drop below zero.

Justification for method for deriving 1 PAM table

One criticism of the method used by Dayhoff for deriving the frequencies at 1 PAM concerns the observation set. To collect sufficiently many values frequencies for 1 PAM were derived from observations at higher PAMs, up to 15 PAMs in some cases. Under the model's assumptions this data contains multiple mutations at the same sites. No adjustment was made for this. Instead a proportionality constant was introduced to decrease the number of changes to 1 change per hundred amino acids. This is equivalent to assuming that there were no multiple mutations. One justification for this approximation is that multiple mutations at 15 PAMs are relatively infrequent. The graph relating PAMs and percentage difference indicates that about 10% of changed residues will have experienced multiple changes at 15 PAMs. Errors from this procedure are in fact substantially lower than 10%. This can be shown by application of the binomial theorem for matrices.

To apply the binomial theorem we re-express the matrix for transition probabilities. A, and hence also M, has most of its weight on the leading diagonal. M can be expressed as:

$$M = I+\epsilon$$

Where I is the identity matrix and ϵ is small. This reflects the fact that at 1 PAM most residues stay unchanged. Entries in the matrix ϵ are of the order 0.01 since the table A corresponds to 1 change per hundred residues. Applying the binomial expansion we have:

$$M^N = (I+\epsilon)^N = I + N\epsilon + \ldots$$

Dayhoff's use of a proportionality constant to reduce 15 PAMs to 1 PAM amounts to deriving $I+\epsilon$ from $(I+\epsilon)^N$ using the first two terms of this approximation. The omitted terms and hence errors in M in using this approximation are thus of the order ϵ^2.

Explanation for symmetry of A'

Dayhoff gives no explanation for why the table A' should be symmetric. A straightforward calculation of the higher PAM tables produces tables which are asymmetric. This is a result of rounding errors in calculation. When raising matrices to high powers small errors in calculation are magnified. Precise calculation should give symmetric tables. To prove the assertion that A' should be symmetric we use the symmetry of A and some algebra:

$$M.\mathrm{diag}(f_j) = A = A^T = (M.\mathrm{diag}(f_j))^T = \mathrm{diag}(f_j).M^T$$

So

$$\begin{aligned} A' &= M^N.\mathrm{diag}(f_j) \\ &= M^{N-1}.\mathrm{diag}(f_j).M^T \\ &= M^{N-2}.\mathrm{diag}(f_j).(M^2)^T \\ &= \quad \mathrm{diag}(f_j).(M^N)^T \\ &= (M^N.\mathrm{diag}(f_j))^T \\ &= (A')^T \end{aligned}$$

Calculation of A'

A program "Dayhoff" was written to compute the Dayhoff frequencies and scoring tables. Calculations were performed to 12 significant figures accuracy. To ensure symmetry of A' the computation of powers of M was arranged to reduce the number of matrix multiplications involved. Even so asymmetrical tables resulted. An ad hoc way used by other programs to fix this is to average the resulting matrix with its transpose. A better way is available. Provided N is even we can use the following identity in the calculation of A':

$$A' = M^{N/2}.\mathrm{diag}(f_j).(M^{N/2})^T$$

This guarantees symmetry. The right hand side of this identity is one of the intermediate steps in the proof of the symmetry of A'. The program was modified to use this form. Of four-hundred values in the Dayhoff scoring tables the corrections to PAM tables at 100 or 250 PAMs, the normal distances used in practice, were slight. Eight entries were changed. These changes affect the scores of protein matches by less than one percent.

Discrimination of similarity scoring tables

Ultimately the justification for the Dayhoff model is empirical. It is a sensitive scoring method. More precisely the Dayhoff scoring scheme has good discrimination between sequences that are related to a query sequence and those sequences which show similarity as a result of chance matching. The sensitivity was shown by Dayhoff by taking scores for comparison of related sequences and comparing these to comparison scores for large numbers of random sequences. In that analysis both alignment scores, and scores for fixed length words were considered. For both methods the Dayhoff table generally separated the signal from the noise distribution by a larger number of standard deviations than did other tables based on exact matching or the genetic code (Schwartz & Dayhoff, 1978). Feng *et al.* (1985) essentially confirmed these results though they noted that for detecting very closely related sequences the identity matrix, that is scoring for exact matches, gave better discrimination.

Analytic measure of discrimination

Discrimination can also be investigated by following an analytic approach. To demonstrate the method we apply this analysis first to scoring that counts only exact matches. In this discussion on analytic measures of discrimination, we look at the expected score and variance of the score of a word of fixed length K. In this we provide an analytic analogue of one of Dayhoff's method for measuring the discrimination of different tables by simulation.

Two words of length K from unrelated protein sequences will have expected identical matching of 0.07 K. The distribution of matching scores for such words is binomial and for large K is approximately normal with variance $0.07\ (1 - 0.07)\ K = 0.065\ K$. In general any scoring scheme that sums K component scores of identical independent distribution will be, by the central limit theorem, approximately normal. Since we know the distribution we can compute the expected significance in standard deviations of a signal level match in terms of its deviation from the distribution of noise level scores. For word pairs with a fraction P of matching residues the significance S of the match in standard deviations is given by:

$$S = (PK - 0.07K)/(0.065K)^{1/2} = (4P - 0.28)K^{1/2}$$

This agrees with the significance estimate arrived at by Brennan *et al.* (1986) by computer comparison of random sequences. The analytic derivation is much more direct.

For DNA sequences of average composition the corresponding mean matching frequency is 0.25 x 3K = 0.75K and the variance 0.25 x (1-0.25) x 3K = $(0.75)^2$K. The factor 3 arises because 3 bases code for one amino acid and K still measures sequence length in residues. The equation for DNA sequence comparisons analogous to the one just given for proteins is then:

$$T = (3QK - 0.75K)/0.75K^{1/2} = (4Q - 1)K^{1/2}$$

Here Q is the observed proportion of matching bases. T measures how far Q, this observed proportion of matching, is from the mean and is in standard deviations.

For unrelated unbiased DNA sequences we have Q=1/4 and T=0 S.D. If instead the two DNA sequences are identical we have Q=1 and T is then 3.0 $K^{1/2}$ S.D. Comparing the protein translation of identical sequences we have P=1 and S=3.7 $K^{1/2}$ S.D.

Proteins with 100% identity (S=3.7) can arise from DNA sequences with 66% identity (T=1.64) since, for many codons, changing the third base does not change the amino acid encoded. These numbers give a numerical indication of the improvement in discrimination between signal and noise that is possible if DNA which codes for protein is translated before comparisons, even where scoring is based on identities only.

More discriminating PAM tables

To extend the analysis of discrimination to arbitrary amino acid scoring tables we make use of the Dayhoff frequencies of substitutions. Let W_{ij} be the score for a match of residue type i against type j. The random matching score X for pairs of amino acids has mean value:

$$E(X) = \Sigma_i \Sigma_j f_i f_j W_{ij}$$

and Variance:

$$Var(X) = \Sigma_i \Sigma_j f_i f_j (W_{ij})^2 - (\Sigma_i \Sigma_j f_i f_j W_{ij})^2$$

And the expected significance for related sequence is then:

$$Z=\frac{\sum_{i=1}^{i=20}\sum_{j=1}^{j=20} A'_{ij}W_{ij}-\sum_{i=1}^{i=20}\sum_{j=1}^{j=20} f_i f_j W_{ij}}{\sqrt{\sum_{i=1}^{i=20}\sum_{j=1}^{j=20} f_i f_j (W_{ij})^2-\left(\sum_{i=1}^{i=20}\sum_{j=1}^{j=20} f_i f_j W_{ij}\right)^2}}$$

Substitution of W_{ij} for exact matching shows that the exact matching matrix gives greater significance scores than the corresponding Dayhoff matrix below 100 PAMs (figure 3.2). This provides an analytic corroboration of Feng *et al.*'s empirical observation on separating very strongly related sequences from noise similarities.

An advantage in having an expression in terms of the W_{ij} for the discrimination now becomes apparent. We can use mathematical techniques to choose new W_{ij} which give the optimal expected Z score. That is, on the assumption that the frequencies A'_{ij} and f_i are correct, we can choose a scoring scheme W_{ij} with maximal discrimination.

Maximization of Z with respect to the W_{ij} is conveniently achieved using the method of Lagrangian multipliers (Arfken, 1985). We observe first that the W_{ij} can be multiplied by a constant without affecting Z. This allows us to introduce the constraint on the W_{ij} that:

$$\mathrm{Var}(X) = 1$$

The maximum is unaffected. We now maximize Z by maximizing the numerator in the equation for Z subject to the constraint. Letting Lambda be the undetermined Lagrangian multiplier we require:

$$\frac{\delta}{\delta W_{ij}}\left[\sum_{i=1}^{i=20}\sum_{j=1}^{j=20}(A'_{ij}-f_i f_j)W_{ij}\right]=$$
$$\lambda\frac{\delta}{\delta W_{ij}}\left[\sum_{i=1}^{i=20}\sum_{j=1}^{j=20} f_i f_j (W_{ij})^2-\left(\sum_{i=1}^{i=20}\sum_{j=1}^{j=20} f_i f_j W_{ij}\right)^2\right]$$

So:

$$(\acute{A}_{ij}-f_if_j) = 2\lambda f_if_j \left[W_{ij}-\sum_{i=1}^{i=20}\sum_{j=1}^{j=20} f_if_jW_{ij}\right]$$

We observe that Z is also unaffected by addition of any constant to all W_{ij} so we are free to set:

$$\sum_{i=1}^{i=20}\sum_{i=1}^{i=20} f_if_jW_{ij}=0$$

Whence:

$$W_{ij}=\frac{\acute{A}_{ij}-f_if_j}{2\lambda f_if_j}=\frac{1}{2\lambda}\left(\frac{\acute{A}_{ij}}{f_if_j}-1\right)$$

Although we can readily determine λ by substitution in the constraint equation, we do not need to. Z is unaffected by addition of a constant to all W_{ij} or multiplication of all W_{ij} by a constant. We get the same maximal Z, i.e. the maximal discrimination with:

$$W_{ij}=\frac{\acute{A}_{ij}}{f_if_j}$$

Thus the odds ratio of Dayhoff gives the optimal discriminating scoring according to the two parts of her model. Taking the log of the odds as the scoring matrix, as Dayhoff does, decreases the discriminatory power of the scoring scheme. A graph comparing the discriminatory power of odds ratios, the log odds and the identity scoring is given in figure 3.2. Additionally the graph shows a fourth curve that is a compromise on the optimal scoring table. The reason for making this compromise is given in the next section.

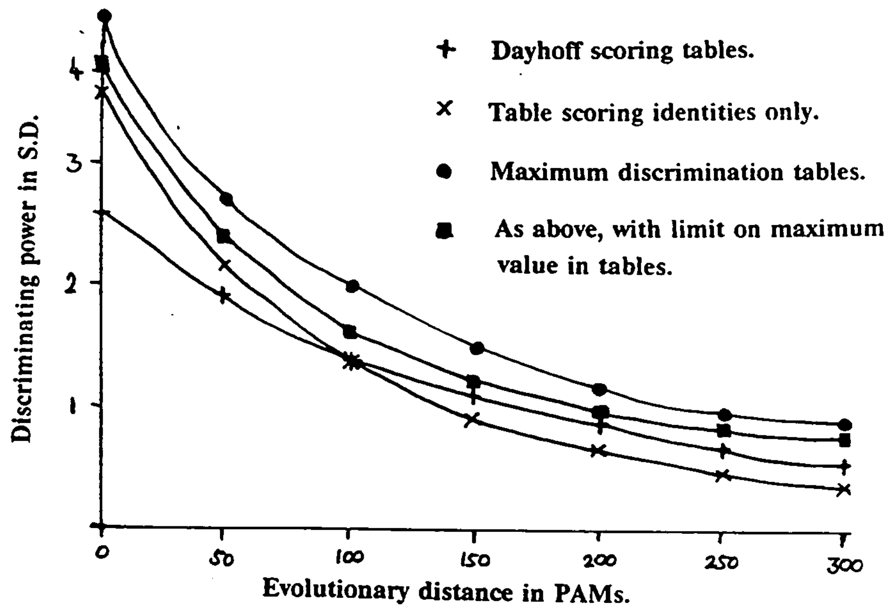


Figure 3.2: Graph showing discriminatory power against evolutionary distance in PAMs.

Disadvantage of the odds table

Use of the odds ratios rather than log odds has a disadvantage. The tables give extreme weight to pairings of amino acids that are unlikely by chance. The new score matrices are thus more prone than Dayhoff's to cause problems where composition deviates from the average assumed in the model. To overcome this a cutoff can be incorporated that limits the maximum value in the scoring table. This reduces the potential for high matching scores to arise from only a few residues in the match.

Limiting high values in the score table through use of a cutoff reduces the expected significance for signal matching when measured against the distribution for noise matching. However, the expected significance scores are still higher than for log odds tables.

Validation

To validate the new tables, a method of testing that reflects the intended use was developed. The test involved comparison of the human beta haemoglobin protein sequence to sequences in a database of 6000 proteins (PIR 23, verified section), which contained 330 globin sequences. Comparison used the Type III algorithm. Since the Type III algorithm requires integer scores with negative expectation, the odds tables were scaled up by a a factor of 4 and a constant offset of 5.5 was subtracted. This gives an expected score of -1.5 per residue for comparison of unrelated sequences.

The table in figure 3.3 shows the number of globins which scored greater than the first non-globin. This gives a measure of the scoring scheme's ability to recover sequences related to a query from a protein sequence database.

	100 PAMs	150 PAMs	200 PAMs	250 PAMs	300 PAMs
A: Odds	296	299	298	291	289
B: Lodds	292	298	298	298	297

Figure 3.3 Table showing number of globin sequences recovered before first non-globin sequence. A: With odds scoring tables. B: With the usual log odds tables. Target; recovery of 330 globin sequences before first non-globin sequence.

In practice the new tables have performance that is only marginally different to the original Dayhoff tables. According to the Dayhoff model there should have been an improvement from using the modified tables. This suggests that the Dayhoff model does not fully capture the structure of signal and noise in the database and that deviations from the Dayhoff model for substitution frequencies are a more serious source of loss of discrimination than the choice of method for combining frequencies to produce scores.

There is little reason to use the new tables. They are no better in practice than the Dayhoff tables which have been used successfully for many years. They introduce an extra arbitrary parameter and, whereas the sum of individual Dayhoff scores has a ready interpretation as a logarithm of odds, the sum of scores from the modified tables do not.

Other amino acid similarity scoring tables

Many indices have been devised for comparing amino acids (Nakai *et al.*, 1988). These are based on measured chemical and physical properties. Scoring schemes for scoring amino acid similarity based on differences of the indeces do not take account of the mutational process. Such scoring schemes are rarely used for protein database searching. Taking account of mutational change improves detection of similarities arising from divergent evolution from a common ancestor. Similarities between protein sequences suggestive of similar structure and hence function will almost certainly be of this kind. One argument for using scoring tables based on chemical and physical properties instead of the Dayhoff tables is that potentially we might detect other functionally important sequence similarities that have arisen by 'convergent evolution'.

With convergent evolution, the same enzymatic function is achieved by evolution from very different ancestral sequences. Two proteins that have achieved similar functions by convergent evolution may have similarities in the three dimensional positioning of their interacting residues at the active sites with this positioning of residues being achieved by radically different folding of the proteins. Residues equivalent in the folded structures would then have markedly different order and spacing in the linear protein sequences. The two classes of serine protease, representatives being subtilisn and chymotrypsin provide an example of this (Kraut, 1977). Both classes of protease have essentially the same mechanism of action. Residues that are functionally equivalent can be identified. In the linear sequences, these equivalent residues are not flanked by local regions of sequence similarity. The kind of structural similarity the two classes of protease show cannot be detected from the sequence data.

If convergent evolution cannot be detected by sequence comparison alone, then it is correct to use a scoring scheme, such as Dayhoff's, based on divergent evolution to score sequence similarity. Using such a scoring scheme can give a sensitive method for finding weakly related sequences against a background of noise.

Chapter 4: Rapid and Sensitive Database Searching

The sensitivity of the Type III NWS sequence comparison algorithm makes it the method of choice for comparing pairs of proteins. The documentation for the GCG sequence comparison software suite states:

> "Bestfit [the local homology algorithm] is the most powerful algorithm we know for identifying the best region of similarity between two sequences whose relationship is unknown." (Devereux *et al.*, 1989a)

Comparison of two proteins using the Type III algorithm produces an alignment and a similarity score. The algorithm can be used for searching a protein database to find if any sequences in the database are similar to a 'query' sequence. Comparison of the query to each protein in the database produces a list of alignments and similarity scores. This list is sorted by score and the high scoring similarities printed out in order of rank, highest scoring first. As well as printing the high scoring sequences' names and their scores for comparison to the query, alignments of the regions which show similarity can be printed. The alignments contain qualitative information whereas the scores are purely quantitative.

Examination of the alignment may show a similarity is less convincing than the score suggests. The similarity may be due to a section of unusual amino acid composition in both query and database rather than to a conserved pattern. Such a similarity may provide less evidence for functional relationship than more heterologous matches of equal score do. This particular artifact of the scoring scheme is a case of the assumption of 'independence of sites' implicit in any amino acid scoring scheme, breaking down.

Because the similarity measure is imperfect, it is usual to present not just results which are clearly signal but also some of the highest scoring results which are likely to be noise. Amongst these, the biologist may spot an alignment with a protein which for biological reasons is more significant than the score alone suggests. A biologist may have greater interest in a similarity low down the list than one high up. For example, if the query protein is differentially expressed in males and females then the biologist may attach greater importance to matches of the query to sex specific sequences from the database.

Sensitivity and selectivity

Two words, 'sensitivity' and 'selectivity' are used in some papers on sequence comparison in describing the quality of the searching performed by an algorithm. 'Sensitivity' is used to mean the ability to detect weak signals. 'Selectivity' is used for the ability to reject noise matches. In producing a list of similarities sorted by score, selectivity can be improved at the expense of sensitivity simply by printing fewer of the results of the search. 'Sensitivity' and 'selectivity' are thus different aspects of one quality, the ability of a sequence similarity scoring scheme to distinguish between signal matches and noise level matching.

Computational cost

In comparing a protein sequence against all sequences in a database many thousands of pairwise comparisons are performed. There is a widespread belief that the sensitive NWS pairwise method of comparison is too computationally expensive to be used in this way on serial computers.

> "The Needleman-Wunsch method is too slow for complete database searches to find sequences that are homologous to an input sequence."
> "Use of the Needleman-Wunsch method to compare two sequences (of length 350 residues) took several minutes of machine time and more time is required for longer sequences." (Mount, 1988)

Consequently alternative solutions are sought. Various methods which are acknowledged to be less sensitive are used instead (Pearson, 1990). This approach is less than ideal. Ultimately the sensitivity of a method is more important. One must consider the investment in time in experimental work to determine the sequences. Lyall states:

> "The real solution to this problem is to use a computer that is powerful enough to run the exhaustive algorithm as a matter of routine. As well as answering a real need, the use of such a machine may well catalyse the development of new methods for the understanding of biological sequences." (Lyall, 1988)

Lyall's work shows just how successful such an approach can be. He used an ICL Distributed Array Processor (DAP) parallel computer which had 1024 simple processors. DAP computers are now manufactured and distributed by Active Memory Technology Limited (AMT, Reading U.K.). Lyall developed a fast parallel implementation, "Prosrch", of the Type III algorithm to run on the DAP. In his doctoral thesis he presents six interesting and unexpected sequence similarities found using "Prosrch".

Lyall also compared the performance of this software to software for similar purposes running on various other machines. The use of the DAP turns out to be cost effective particularly when compared to software on vector supercomputers. The lesser cost effectiveness of such machines can largely be attributed to the presence of special purpose hardware for floating point calculations. This hardware adds considerably to the cost of the machines whilst not materially contributing to speed of operation in this application.

Development of the early version of "Prosrch" which had a speed of 600,000 PMEs^{-1} has continued with improvements being made by Collins to increase speed to 7,000,000 PMEs^{-1} and to facilitate investigations of the 'twilight zone' between signal and noise matching (J.F. Collins, ICMB Edinburgh, personal communication). Powerful hardware is clearly one solution to the problem of combining speed with sensitivity. A disadvantage of parallel machines is that they are not widely available.

Database reduction

One alternative way to increase speed is to reduce the size of the database being searched. For example a database containing only representative members of each sequence family could be searched. This inevitably reduces the ability to detect weaker matches. Members of a family will show a range of values of similarity score against any given query. The method works well when searching with a sequence similar to the representative, less well when searching with a sequence most similar to one of the more distant family members.

Rather than choosing a representative protein for each family, modified sequences that attempt to capture the essential aspects of similarity shared by all members of the family could be used. The use of such 'derived patterns' gives an alternative method of database reduction.

Derived patterns

Using derived patterns for database reduction is possibly even less satisfactory than choosing representative members, if the goal is to make new discoveries through sequence comparison.

One of the sequence similarities reported by Lyall that was found using "Prosrch" is indicative of the problem. A yolk amino acid storage protein (YP3) in fruit fly, *Drosophila melanogaster*, was found to be similar to a lipase from the liver of pig, *Sus scrofa* (Garbedian *et al.* 1987). A serine residue at the active site is crucial to the lipase's lipid cleaving activity and is present in all members of the lipase family. This residue would be part of a derived pattern for lipases. In YP3, the residues in the corresponding position is glycine. This lead to a hypothesis that YP3 is not a lipase, that it binds to but does not cleave a lipid analogue, a precursor to a hormone called ecdysone. This hypothesis was confirmed by experiment. The interpretation is that as YP3 is broken down to release its amino acids to the growing larva, controlled quantities of hormone are released. Had the computer search required matching of the active site serine residue the search would not have detected the similarity.

'Prosite'

Databases of derived patterns are being actively developed. Though caution is needed in their use, they are a valuable method for organising information about sequences. The most comprehensive catalogue of derived patterns so far developed is the 'Prosite' motif directory (Bairoch, 1989a). This exists in both a printed and an electronic version. In the 'Prosite' directory families of proteins are catalogued. For each family a 'motif pattern' is given. The motif patterns of 'Prosite' represent 'key residues'. These key residues are residues conserved or substituted for by similar amino acids in all examples of a family. An example of a 'Prosite' motif for the Pentraxins, a family of serum and plasma proteins some of which are expressed during acute phase response to injury, is shown below:

```
Pentraxin family signature
     HXCX(S,T)W
```

The bracketed residues indicate alternative possibilities at one site. An X indicate a site at which any residue is acceptable. The patterns are short, simple and straightforward to interpret. They are intended to be diagnostic for family membership.

The 'Prosite' motifs make searching for membership of families extremely rapid. The database of patterns is much smaller than the sequence database. Release 4 of 'Prosite' contains 198 protein motifs with an average of five residues in each motif pattern. As well as the small size, the fact that 'Prosite' contains highly conserved residues makes fast exact matching of patterns more acceptable than it would be were the selected residues more variable.

Insensitivity of 'Prosite' patterns

A criticism of the 'Prosite' directory is the insensitivity of most of the patterns. With the protein family cytochrome c, members of the family shows strong homology throughout their length, that is over a stretch of some 100 amino acid residues. In the 'Prosite' directory, a motif pattern of 5 amino acids is chosen near to a haem attachment site. Whilst this pattern may be superior to other possible short patterns, the use of a short pattern means that much of the information is discarded.

The loss of information arising from using short patterns affects detection of new members of a family. Whilst careful choice of motif can make the pattern diagnostic for family membership for all data available at the time the pattern was chosen, the motifs will not necessarily be so successful on new examples not available when the patterns were designed. This is indicated by the fact that the patterns are changed to accommodate new data. A fission yeast cytochrome has a leucine in place of a methionine that is conserved in all the other known cytochrome c's. A change in the motif pattern for cytochrome c was necessary to take account of this new special case and this is documented in 'Prosite'. For smaller families the range of variation at sites is less well determined. For these families there is therefore less confidence that the chosen motif pattern, diagnostic for all current examples, will continue to discriminate well with new examples.

Measuring information content of 'Prosite' patterns

The shorter patterns match against unrelated proteins more readily than longer patterns would. From a table of amino acid frequencies such as the one in figure 1.2, it is straightforward to calculate the likelihood of matching random sequence with any motif. One way to express this is as the information content of the pattern measured in bits, that is, the *Log* to base 2 of the probability of matching (Shannon & Weaver, 1949). The motif patterns for 'Prosite' functional sites have a wide range of information content from 8.6 bits (cell attachment sequence), to 49.3 bits (nuclear hormone receptors' DNA-binding region signature). The former pattern would be expected to occur by chance every $2^{8.6} = 300$ residues, i.e. 7,700 times in a typical database. One would expect to search 200,000,000 databases to find the latter pattern by chance.

The problem of poor diagnosis by pattern has been explicitly recognised in the directory for the 'leucine zipper motif' (information content 14.2 bits, expected 160 times in a single database search):

"As this is far from being a specific pattern you should be cautious in citing the presence of such pattern in a protein if it has not been shown to be a nuclear DNA-binding protein." (Bairoch 1989b)

The problem of low information content is a general one that arises from having few residues in each pattern. It is not restricted to this one example. There is a danger that features postulated by pattern homology come to be treated as genuine. One feature (information content for 'Prosite' pattern, 16.8 bits) that is notorious for this is the 'zinc finger' (Frankel & Pabo, 1988). The dangers would be more obvious were a clearer indication of the specificity of each pattern, either as a probability of random matching or in terms of information content, given for each motif.

Use of 'Prosite'

'Prosite' does not represent an acceptable alternative to rapid and sensitive database searching such as is provided by "Prosrch". Its function is instead to annotate a new protein sequence with suggestions of sites with already known and characterised function. Its role is complementary to full sequence database searches.

Approximate methods for sequence comparison

Since NWS algorithms are computationally expensive, to make searching possible at reasonable speeds on serial machines, faster methods which are less sensitive have been developed (Pearson, 1990). The most popular of these, "FastP", compares 800 protein pairs per minute. These more rapid programs are invariably based on exact matching of words.

"FastP's" two tier approach

To overcome some of the sensitivity drawbacks of word based matching "FastP" uses a two stage process. The initial search, based on exactly matching words, acts as a filter. This picks out proteins for a more sensitive method to examine. The filter reduces the workload of the sensitive method to a manageable level.

In "FastP's" two tier approach the first stage, the initial filtering, may discard matches which would score highly under the more sensitive method. A match that would be accepted in the more sensitive stage is not guaranteed to pass through the coarser first stage and could be lost. In this sense the two stages are not fully compatible.

The risk of missing a good score can be reduced by letting more results pass through the filter to the second stage, that is by reducing the stringency of the filter. There is no criterion though for how to set the stringency to guarantee that all high scores will be found. For a discriminating comparison either a more sensitive method must be used throughout, or a filter must be devised that cuts down the workload but which is guaranteed to pass on all matches which would be recognised as 'good' by a second stage.

"FastP's" second stage

The second more sensitive stage of "FastP" performs an NWS-like alignment to refine the score. This stage uses exactly matching words rather than individual residue matches. As a result far fewer possible alignments need to be considered. Additional speed benefits arise from using a dictionary just as they did in the filtering stage. Even if filtering parameters are set to pass all proteins to the more sensitive stage, the final scoring is still based on exactly matching words.

A later development of "FastP" called "FastA", improves the second stage by scoring words using Dayhoff scores in the more sensitive phase. In addition it overcomes a major defect of "FastP", that "FastP's" first stage was a global rather than a local algorithm. Previously, in longer sequences, a smaller region of good matching could be obscured if there were insufficient word matches in it to be significantly above average for sequence comparison of entire sequences.

The "FastP" algorithm is asserted to have running time that depends on the sum, rather than the product, of the lengths of sequences compared (Wilbur & Lipman, 1983). In fact, since the number of matching word pairs of a particular fixed length is proportional to the product of sequence lengths, "FastP" has time complexity $O(n^2)$. However, the "FastP" algorithm makes considerable savings in comparison to the Type III algorithm, which is also $O(n^2)$. The use of exact matching words of length 2 leads to an approximately 200 fold reduction factor in the work performed, whilst maintaining a useful level of sensitivity. This makes the algorithm very suitable for rapid database searching and "FastP" is in widespread use.

Theoretical advances in comparison algorithms

The DAP program indicates the importance of methods which are both sensitive and rapid. Theoretical advances might lead to algorithms that perform with the rapidity of "FastP" and the sensitivity of the NWS algorithm on serial machines. Theoretical work on the NWS algorithms shows that theoretical speed improvements to the algorithm are possible, in particular improvements that lead to the 'four Russians' version of the algorithm (Masek & Paterson, 1983). This algorithm works by first calculating a large number of 2 by 2, 3 by 3 or more generally K by K alignment path matrices. These are combined in producing a path matrix for the complete alignment. The algorithm takes time $O(n^2/Log\ n)$ rather than the normal $O(n^2)$.

Using 1 by 1 submatrices the algorithm is equivalent to the normal implementation and there is no gain. With $K = 2$ precalculation of around 10^{10} submatrices is required. This estimate is based on 20 amino acids, a 100 PAM table and an indel penalty of 10. Storing these submatrices in main memory is impractical on all but the largest computers. For the method to give any gain at all, retrieval from disk of part results would need to be faster than recalculation. The authors of the paper on this method do not mention a practical implementation of this algorithm.

The four Russians approach gives a theoretical gain which seems to be of no practical use whatsoever for protein database searching. *Log n* grows slowly with n, so n must be very large before we can hope for an appreciable gain. As a minimum n must be large enough to compensate for the overheads in precalculating the submatrices. In practice these overheads are colossal. Not only is the time needed in calculating the submatrices large, the storage requirements for the matrices are large too. There is an exponential increase in number of submatrices with increase in submatrix size, alphabet size, indel penalty and range of score in the scoring table. For protein database searching, using reasonable parameters, the four Russians method is impractical.

Concluding remarks

Speed and sensitivity are important in protein database searching. Approaches to achieving these apparently conflicting goals have been taken by a number of authors with varying degrees of success. These include database reduction, use of word based methods, use of powerful computers and fundamental changes to the algorithms for NWS string comparison. A fifth approach, taken in this work, recognises the potential for improved serial implementation of the Type III algorithm. This resulted in an extremely rapid serial implementation. This is described in the next chapter. This algorithm is of similar speed to "FastP", runs on a microcomputer and has the same sensitivity as the DAP "Prosrch" algorithm.

Chapter 5: Techniques to Get More from Machines.

In the previous chapter we looked at various approaches to rapid and sensitive database searching. Here we present a new approach that uses new implementation techniques. These give a fifty fold speed improvement to the serial Type III algorithm. The new implementation's importance is that it makes the Type III algorithm run rapidly on widely available machines, that is on personal microcomputers (PCs) such as those manufactured by International Business Machines. In later chapters we discuss applications that were built around this software.

As well as describing the new techniques used for rapid sequence comparison, this chapter describes other implementation issues encountered in the study. The chapter is divided into sections covering specific problems. Each section starts with a statement of the problem, is followed by a description of the methods used to tackle the problem and finishes with a discussion. The methods sections concentrate on the principles behind the methods used. Further detail of methods are contained in appendices where appropriate.

Type III comparison (Speed)

Problem:

A crucial problem with a standard implementation of the Type III algorithm is its low execution speed. Apart from the use of parallel computers, other techniques to improve speed make some compromise on the sensitivity of searching in order to obtain their speed.

"The results of searches with FASTA compare favourably with results using NWS-based programs that are 100 times slower. FASTA is slightly less sensitive..." (Pearson, 1990)

The problem tackled here is to find techniques that increase the speed of the Type III searching on serial machines.

Methods:

A combination of techniques, rather than any one technique on its own, were central to the speed improvements. Although each saving may appear to have a marginal effect, the combined effect is significant. Savings in one part of the algorithm only have a marked influence on the overall speed when other inefficiencies are removed. Because the path matrix calculations involve operations performed thousands of millions of times, apparently small savings can have a large effect on the algorithm's running time.

The first step in improving performance uses the following observation: Any two columns of the path matrix with the same sequence character at their head will have the same one step diagonal scores. Production of a table of these 23 possible columns of scores gives a substantial saving in the number of indexing operations the algorithm performs. Using this intermediate table, the number of indexing operations in retrieving one step diagonal scores is halved. The row index is used to directly access scores from one of the 23 columns in one indexing operation, rather than first retrieving a sequence character (first indexing operation), and then using the sequence character as an index into the amino acid score table (second indexing operation).

The second optimisation is more complex. It rests on noticing that many of the entries in the path matrix hold zero scores. This is true for over two thirds of the entries. This arises because the scoring tables have a negative expectation. The majority of paths score negatively and so have scores reset to zero at each step.

Reorganisation of the order of operations is required. The Type III algorithm is normally presented as in Chapter 2, in terms of how values at each cell are computed. After re-expressing the algorithm in terms of how each cell value influences its neighbours, the fact that zero scores do not influence other cell values can be properly exploited. Zero scores can then be processed rapidly. A second saving arises from the new order of calculation. Provided that neither a negative score nor a zero score influences other cell values, negative scores do not need to be converted to zero.

For efficiency the software is written in assembly code. At this level of expressing an algorithm, fetches of information from memory to processor and storage of information from processor to memory are made explicitly. Deferred assignment of values held in registers to variables held in memory is a technique that can sometimes be used to reduce the number of times values in processor registers are written out to memory. This optimisation technique normally relies on a guaranteed pattern of memory reads and writes. The effect of a conditionally executed statement can disrupt this, and does so in the Type III algorithm. However, a new technique was developed to extend the method of deferred assignment. This new technique, 'conditional deferred assignment' is described in Appendix 3. It recaptures the ability to have a known history of memory interactions and uses multiple versions of the code to do this.

The final important optimisation step is to reduce loop overheads for processing one column. The determination of maximum score in a column which could be done in a separate loop is instead performed in the main loop. Having one loop instead of two halves the loop overheads. Moreover, the test for maximum need take place only in the infrequently executed code that deals with larger positive path scores. This subdivision of the algorithm into parts for different levels of scoring arose from the previous two optimisations. The testing of the loop counter is removed by using a rogue value (an impossibly high value) to terminate the loop. Testing for the end of the loop only occurs where maximum score may be being updated. This arrangement virtually eliminates loop overheads.

Discussion:

The new implementation of the algorithm performs an average of 200 protein comparisons per minute, a speed of 300,000 PMEs^{-1}. A sensitive database search can thus be performed in under an hour on inexpensive hardware. The machine code algorithm has been built into a database searching program "Prowl", written in Pascal for performing such searches.

The new implementation is the result of successive improvements to the efficiency of an initial version. The idea for an optimisation often arose from simpler optimisations. The rearrangement of the order of calculation, for example, arose from combining two optimisations concerned with low scores.

How is it that the scope for dramatic improvements in speed in the serial Type III algorithm has in the past been overlooked? One possible reason is the practice of using asymptotic measures to measure performance. The improvements described in the preceding section do not affect the asymptotic measure. Asymptotic measures are useful precisely because they are a property of the algorithm rather than the implementation. The example illustrates a danger in relying too heavily on asymptotic measures when dealing with a practical computational problem.

There are precedents for this. Theoretical advances in algorithms for linear programming problems that resulted in an $O(n^6)$ algorithm are not used in practice (Osborne, 1985). The normal simplex algorithm with exponential time worst case performance performs better in practice and is used instead.

The situation for Type III searching is more prosaic. The asymptotic order does not distinguish between variants of an algorithm that differ in speed by a constant factor. An emphasis on asymptotic orders may have lead software developers to overlook the substantially greater efficiency possible in the organisation of calculations in the standard algorithm.

Path reconstruction (Memory space)

Problem:

Generation of comparison scores rapidly is not enough. Alignments as well as the alignment scores are needed. The pressure for rapid computation is not intense since only a small fraction of the sequences compared need to be presented to the user as alignments. Rapid computation here is not crucial. The problem in implementing the reconstruction algorithm is limited RAM memory. On a machine with a large memory it is feasible to store an entire path matrix in main memory. From this the alignment can be reconstructed in steps as described

Uses of multiple alignments

Investigating mechanism.

Multiple alignments are useful in investigations of the functions of individual residues in a protein. They show which residues are variable and which conserved. Residues which are conserved in all the sequences are the most likely to be crucial to the function. These conserved residues may be part of the active site in an enzyme, or they may be vital to the three dimensional structure. Where there is variation in a column of an alignment the kind of variation also gives information. It can suggest the role played by an amino acid. Some columns of multiple alignments, for example, contain only charged residues. The sign of the charge, positive or negative, may vary. Such a site in a protein is likely to have a different role to one for which all substitutions are charged residues of the same sign. Information about variations can thus act as a guide for experimental work to determine the function of individual residues.

The pattern of conservation of residues can give information about cysteine residues. These residues may or may not participate in disulphide bridges with partners elsewhere in the protein. No other residue type can fulfil this role. Loss of a disulphide bridge would be expected to have a marked effect on the structure and stability of a protein. If one sequence in a multiple alignment lacks a cysteine at a particular site which is cysteine in the others, it suggests that the cysteine is used for some purpose which other residues can fulfil and consequently is not part of a disulphide bridge.

An example which shows how multiple alignments can draw attention to structurally important residues is a particular glycine residue which is absolutely conserved in all haemoglobins. Studies of three dimensional structures reveal the explanation. The glycine occurs where two alpha helical structures, the B and E helices cross (Stryer, 1981). No other residue is sufficiently small to fit in the space available.

The information which multiple alignments contain is especially relevant to methods for secondary structure prediction (Zvelebil *et al.*, 1987). The variation at sites shown by multiple alignments gives additional information about structure not present in a single sequence. One example of this is that residues that vary are more likely to be near the surface of a protein than in the core of the protein (Perutz & Lehmann, 1968).

Searching

Computer searches can make use of information presented in a multiple alignment about the range of variations at each site. Smith & Smith (1990), suggest one method that could be implemented using a matrix that gives scores for each amino acid at each site, a 'template matrix'. In the template matrix most weight is attached to residues which are absolutely conserved. Template matrices are computed from multiple alignments, typically by averaging scores for the amino acids in a column. This takes into account the variability at a site and the kind of variation. A template matrix represents a consensus about the patterns shared by the sequences. If previously unknown members of a protein family conform to the consensus pattern of conservation observed in the known examples, then a databse search using template matching will give more discriminating searching for new family members than comparison using any one example from the family could (Taylor, 1986).

One advantage of using a family consensus pattern is that a single comparison of a new sequence against a pattern can be used to check for family membership. It is worth emphasising that this may be less sensitive than comparing a sequence individually with each member of the family in turn. The new sequence may be more similar to the member of the family most similar to it than it is to the consensus pattern for the family. A mental picture for this is to represent the sequences as points. The distance between the points represents the degree of dissimilarity. A family of sequences will form a cluster. The pattern for the family could be represented by a point that is at the centre of the cluster. A new member to the family is, however, likely to be closer to some member of the family than it is to this centre.

Motif patterns such as those of the 'Prosite' directory discussed in Chapter 4, are also derived from multiple alignments. They offer an alternative and less sensitive method for defining patterns of conservation.

Sequence editors

For pairwise alignment, NWS algorithms are the accepted standard. The situation is quite different for multiple sequence alignment. No method has yet become universally accepted. It is not clear how best to extend a scoring scheme that works well for pairwise alignment to score multiple sequence alignments. A second problem is that the natural extensions of the NWS algorithms to score columns of three or more residues, rather than scoring residue pairs, have very high computational demands. To make improvements to existing multiple sequence alignment algorithms involves addressing both purely computational problems, and the problem of defining what good multiple sequence alignment is. The latter requires some knowledge of patterns which actually occur in sequence families.

To gain an understanding of patterns, many alignments for different protein families must be examined. Moreover these alignments should be adjusted to explore possibilities different to those suggested by computer alignment. Patterns which computer analysis misses can only be characterised by manual examination of sequences.

For closely related proteins, alignments can be examined and adjusted on a text editor. For all but the simplest alignments this is time consuming. With a text editor it is easy to delete sequence characters accidentally when removing unwanted spaces from an alignment. There is a need for special purpose multiple sequence editors.

There is a second reason why a multiple sequence editor is important for development of new automatic methods of multiple sequence alignment. A multiple sequence alignment editor can act as a front-end to a new alignment method. It can take care of the loading and the formatting of sequences and the display of results. This simplifies the testing of a new multiple alignment algorithm.

The need for a multiple sequence editor is not specific to investigation of improved algorithms for alignment. Until automatic methods produce entirely satisfactory results manual adjustment of computer generated multiple sequence alignments will continue to be important.

A number of multiple sequence alignment editors have been described in the literature; "Lineup" (Devereux *et al.*, 1984) "Homed" (Stockwell & Peterson, 1987), "Mase" (Faulkner & Jurka 1988), "Esee" (Cabot & Beckenback 1989) and "Alma" (Thirup & Larsen 1990).

Sequence editor on the VAX

Initially work on multiple sequence alignment used the GCG "Lineup" editor running on a VAX computer. With this editor alignments of the lipase / yolk protein family discussed in Chapter 5, and of a family of citric acid cycle proteins were explored.

Fortran source code for "Lineup" was available. "Lineup" was modified by addition of new code to perform automatic multiple sequence alignment using a new method. The new method performed all *1/2 n(n+1)* pairwise alignments of *n* sequences and recovered information about compatible parts of the alignments. Where all alignments agreed, it aligned these residues, regions between being divided into unaligned blocks. The new method proved unsatisfactory for all but the most closely related families of sequences. It depended on low levels of conflict between the pairwise alignments. This only occurred in the straightforward cases, strongly related sequences or parts of sequences, that were easy to align 'by eye'.

The VAX development environment and "Lineup" editor proved to be unsatisfactory for this work. "Lineup" is the largest program in the GCG package consisting of 10,000 lines of source code excluding subroutines shared by other programs in the package, for example subroutines for reading sequence files. To reduce compilation times, compilation was restricted to the new program section, the bulk of the code being pre-compiled. However, linking of the code was still necessary for each change. This took a minimum of three minutes. This contrasts with combined compilation and linking times of a few seconds for modular software written in Turbo Pascal for the IBM PC. As well as having a development environment that greatly facilitated program development, the PC had a second advantage. The PC had the potential for an alignment editor with a better user interface. For the VAX, updates to the screen had to be kept to a minimum as the updates took place over a relatively slow serial line. The PC permitted a much more responsive user interface as well as the use of colour. The speed of response in particular was felt to be important for interactive exploration of many different possible alignments.

Development work moved to the PC. To make this move a new multiple sequence editor for the PC was written. This program was called "Medal" for Multiple sequence EDitor and ALigner. The new editor could be more readily modified to test out new ideas than could "Lineup".

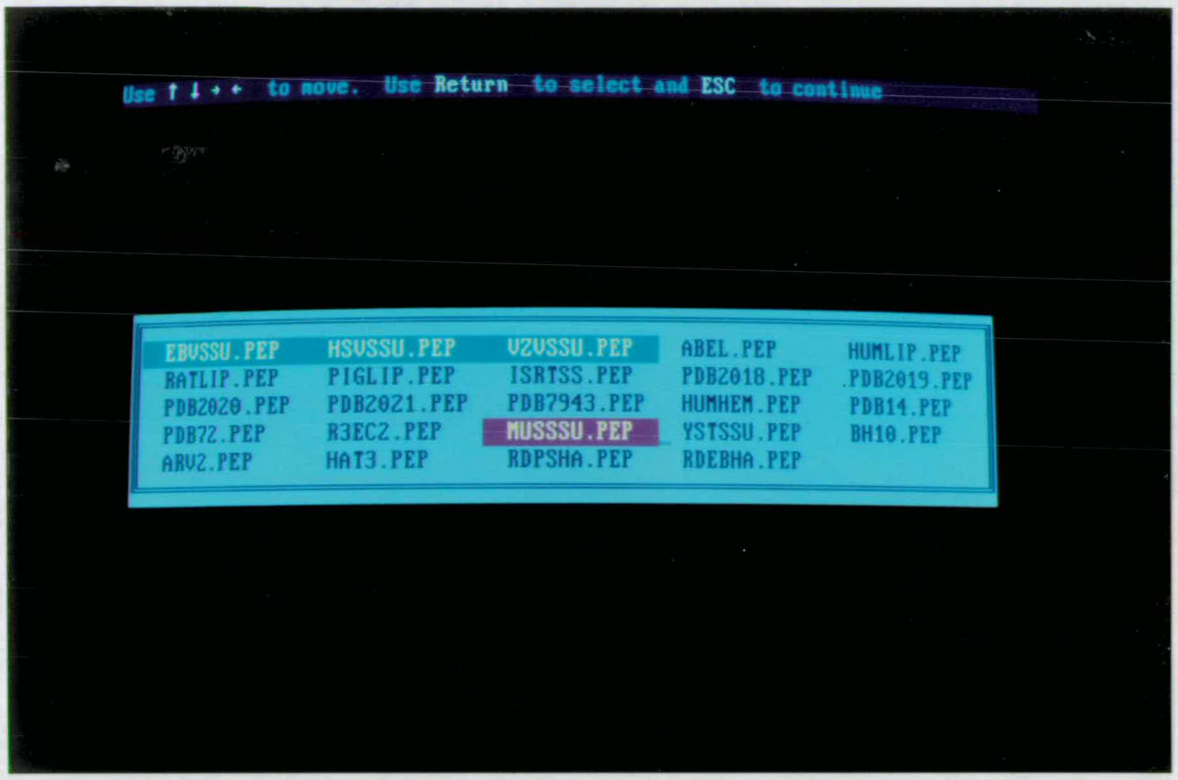
"Medal" has a modular design. It makes use of the Turbo Pascal facility to define software 'units' containing related subroutines and datastructures. This modularity is important in development work. The automatic multiple alignment routine of "Medal" is contained in a separate unit and could be replaced with an alternative one without losing the interface features.

General editor interface features

Some of the interface features of "Medal" are relevant to general editor design and not specific to Molecular Biology. They are not essential for the actual task of sequence alignment editing. Features such as the 'help' facility could easily have been omitted. One reason for developing a good interface to the editor was to encourage use and criticism of the program by molecular biologists.

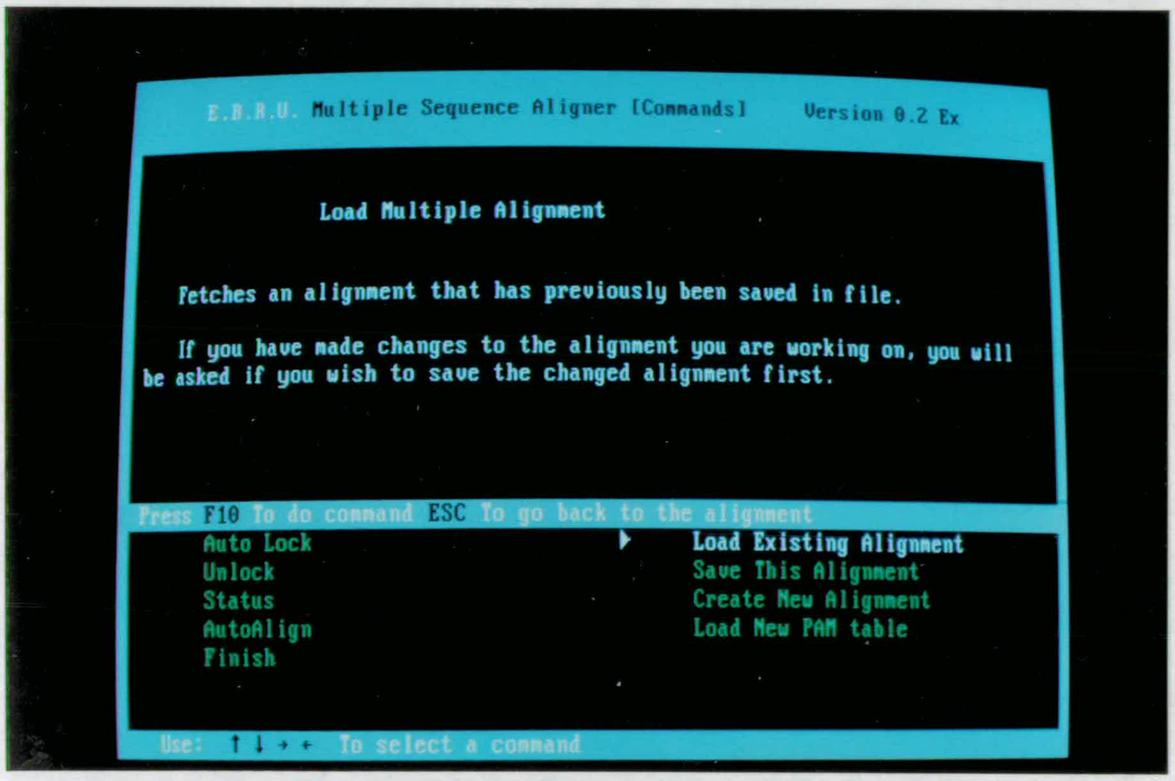
Computer interface design is itself an active field of research (Long & Whitefield, 1989). Rather than performing a thorough study of interface design, designs that worked well in other applications were adapted.

"Medal" has pop-up directory lists from which any individual file can be selected. Pop-up menus are used in many PC programs. In "Medal" the pop-up directory has an additional facility to allow selection of ordered sets of files. This is needed for creation of a multiple alignment from a number of independent sequence files. Pressing 'enter' selects the file currently indicated by a block cursor. Each selected file is moved to the start of the pop-up menu and is highlighted (figure 6.1). Files that have been selected can be deselected in the same manner. The whole set of selected files can then be accepted by another key press.



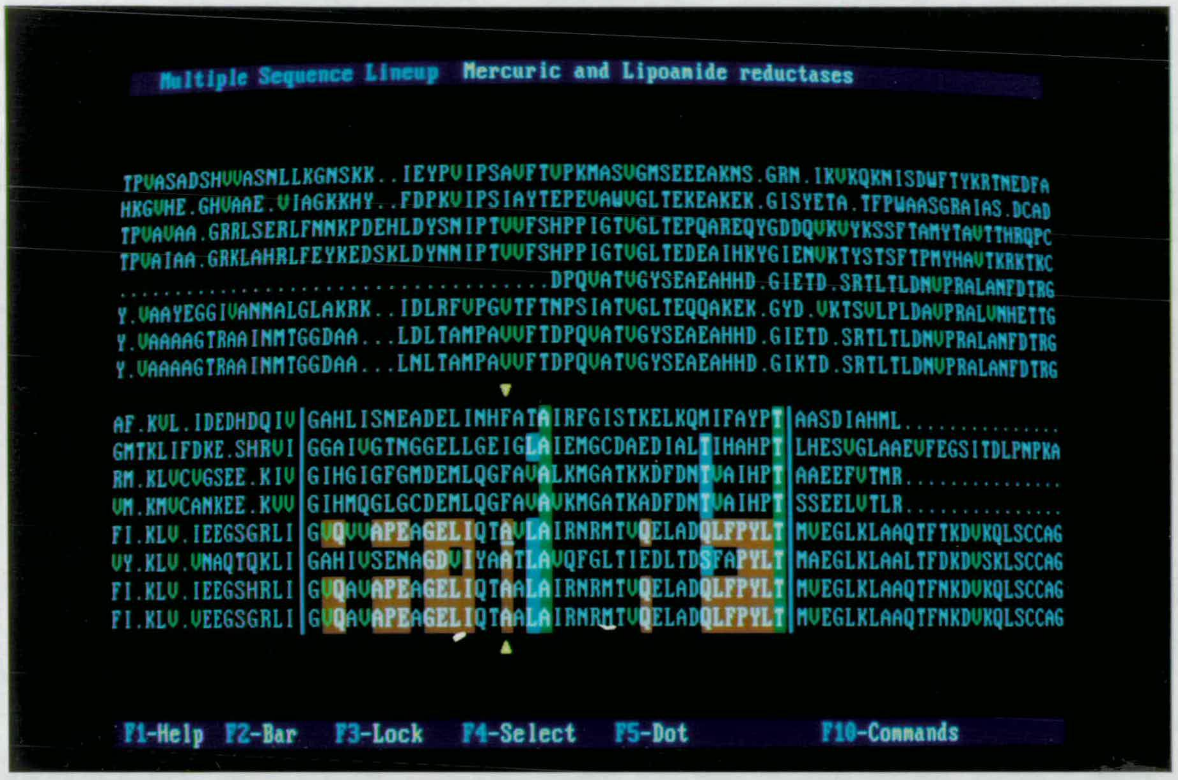
```
Use ↑ ↓ → ←  to move.  Use Return  to select and ESC  to continue

EBVSSU.PEP    HSVSSU.PEP    VZVSSU.PEP    ABEL.PEP      HUMLIP.PEP
RATLIP.PEP    PIGLIP.PEP    ISRTSS.PEP    PDB2018.PEP   PDB2019.PEP
PDB2020.PEP   PDB2021.PEP   PDB7943.PEP   HUMHEM.PEP    PDB14.PEP
PDB72.PEP     R3EC2.PEP     MUSSSU.PEP    YSTSSU.PEP    BH10.PEP
ARV2.PEP      HAT3.PEP      RDPSHA.PEP    RDEBHA.PEP
```

Figure 6.1: Pop-up directory for selecting sequences to make a multiple alignment. The file names in yellow are the selected sequences. The file currently open to selection is indicated by the purple block cursor.



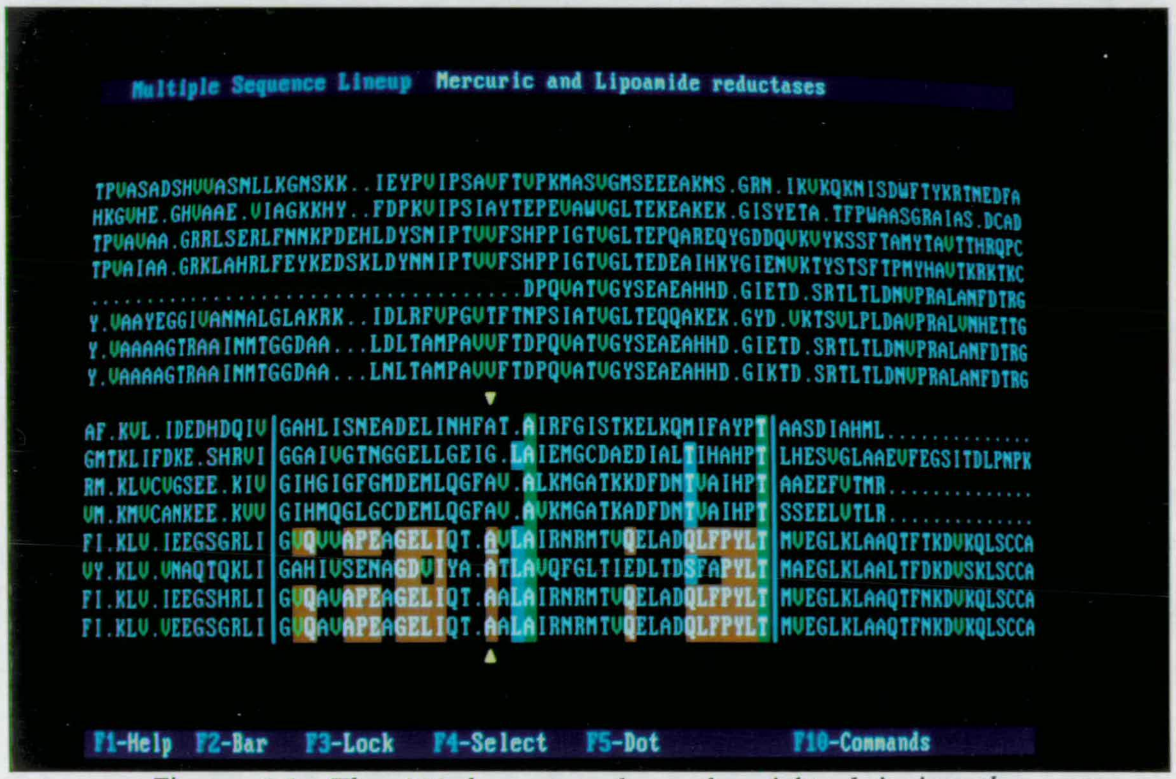
```
E.B.R.U. Multiple Sequence Aligner [Commands]     Version 0.2 Ex

          Load Multiple Alignment

   Fetches an alignment that has previously been saved in file.

   If you have made changes to the alignment you are working on, you will
be asked if you wish to save the changed alignment first.

Press F10 To do command ESC To go back to the alignment
      Auto Lock                       ▶  Load Existing Alignment
      Unlock                             Save This Alignment
      Status                             Create New Alignment
      AutoAlign                          Load New PAM table
      Finish

   Use:  ↑ ↓ → ←  To select a command
```

Figure 6.2: "Medal's" help facility. The command currently selected by the cursor is described more fully in the top two thirds of the screen.



```
Multiple Sequence Lineup  Mercuric and Lipoamide reductases

TPVASADSHVVASNLLKGNSKK..IEYPVIPSAVFTVPKMASVGMSEEEAKNS.GRN.IKVKQKNISDWFTYKRTNEDFA
HKGVHE.GHVAAE.VIAGKKHY..FDPKVIPSIAYTEPEVAWVGLTEKEAKEK.GISYETA.TFPWAASGRAIAS.DCAD
TPVAVAA.GRRLSERLFNNKPDEHLDYSNIPTVVFSHPPIGTVGLTEPQAREQYGDDQVKVYKSSFTAMYTAVTTHRQPC
TPVAIAA.GRKLAHRLFEYKEDSKLDYNNIPTVVFSHPPIGTVGLTEDEAIHKYGIENVKTYSTSFTPMYHAVTKRKTKC
..................................DPQVATVGYSEAEAHHD.GIETD.SRTLTLDNVPRALANFDTRG
Y.VAAYEGGIVANNALGLAKRK..IDLRFVPGVTFTNPSIATVGLTEQQAKEK.GYD.VKTSVLPLDAVPRALVNHETTG
Y.VAAAAGTRAAINMTGGDAA...LDLTAMPAVVFTDPQVATVGYSEAEAHHD.GIETD.SRTLTLDNVPRALANFDTRG
Y.VAAAAGTRAAINMTGGDAA...LNLTAMPAVVFTDPQVATVGYSEAEAHHD.GIKTD.SRTLTLDNVPRALANFDTRG

AF.KVL.IDEDHDQIV |GAHLISNEADELINHFATAIRFGISTKELKQMIFAYPT|AASDIAHML..............
GMTKLIFDKE.SHRVI |GGAIVGTNGGELLGEIGLAIEMGCDAEDIALTIHAHPT|LHESVGLAAEVFEGSITDLPNPKA
RM.KLVCVGSEE.KIV |GIHGIGFGMDEMLQGFAVALKMGATKKDFDNTVAIHPT|AAEEFVTMR..............
VM.KMVCANKEE.KVV |GIHMQGLGCDEMLQGFAVAVKMGATKADFDNTVAIHPT|SSEELVTLR..............
FI.KLV.IEEGSGRLI |GVQVVAPEAGELIQTAVLAIRNRMTVQELADQLFPYLT|MVEGLKLAAQTFTKDVKQLSCCAG
VY.KLV.VNAQTQKLI |GAHIVSENAGDVIYAATLAVQFGLTIEDLTDSFAPYLT|MAEGLKLAALTFDKDVSKLSCCAG
FI.KLV.IEEGSHRLI |GVQAVAPEAGELIQTAALAIRNRMTVQELADQLFPYLT|MVEGLKLAAQTFNKDVKQLSCCAG
FI.KLV.VEEGSGRLI |GVQAVAPEAGELIQTAALAIRNRMTVQELADQLFPYLT|MVEGLKLAAQTFNKDVKQLSCCAG

F1-Help  F2-Bar   F3-Lock   F4-Select   F5-Dot         F10-Commands
```

Figure 6.3: The cursor is on the second sequence on an 'A' residue (To help locate the cursor on the screen there is a pair of yellow triangles to indicate the column). Locked residues are shown with coloured backgrounds. The blue bars prevent changes on one side influencing the alignment on the other side.



```
Multiple Sequence Lineup  Mercuric and Lipoamide reductases

TPVASADSHVVASNLLKGNSKK..IEYPVIPSAVFTVPKMASVGMSEEEAKNS.GRN.IKVKQKNISDWFTYKRTNEDFA
HKGVHE.GHVAAE.VIAGKKHY..FDPKVIPSIAYTEPEVAWVGLTEKEAKEK.GISYETA.TFPWAASGRAIAS.DCAD
TPVAVAA.GRRLSERLFNNKPDEHLDYSNIPTVVFSHPPIGTVGLTEPQAREQYGDDQVKVYKSSFTAMYTAVTTHRQPC
TPVAIAA.GRKLAHRLFEYKEDSKLDYNNIPTVVFSHPPIGTVGLTEDEAIHKYGIENVKTYSTSFTPMYHAVTKRKTKC
..................................DPQVATVGYSEAEAHHD.GIETD.SRTLTLDNVPRALANFDTRG
Y.VAAYEGGIVANNALGLAKRK..IDLRFVPGVTFTNPSIATVGLTEQQAKEK.GYD.VKTSVLPLDAVPRALVNHETTG
Y.VAAAAGTRAAINMTGGDAA...LDLTAMPAVVFTDPQVATVGYSEAEAHHD.GIETD.SRTLTLDNVPRALANFDTRG
Y.VAAAAGTRAAINMTGGDAA...LNLTAMPAVVFTDPQVATVGYSEAEAHHD.GIKTD.SRTLTLDNVPRALANFDTRG

AF.KVL.IDEDHDQIV |GAHLISNEADELINHFAT.AIRFGISTKELKQMIFAYPT|AASDIAHML.............
GMTKLIFDKE.SHRVI |GGAIVGTNGGELLGEIG.LAIEMGCDAEDIALTIHAHPT|LHESVGLAAEVFEGSITDLPNPK
RM.KLVCVGSEE.KIV |GIHGIGFGMDEMLQGFAV.ALKMGATKKDFDNTVAIHPT|AAEEFVTMR.............
VM.KMVCANKEE.KVV |GIHMQGLGCDEMLQGFAV.AVKMGATKADFDNTVAIHPT|SSEELVTLR.............
FI.KLV.IEEGSGRLI |GVQVVAPEAGELIQT.AVLAIRNRMTVQELADQLFPYLT|MVEGLKLAAQTFTKDVKQLSCCA
VY.KLV.VNAQTQKLI |GAHIVSENAGDVIYA.ATLAVQFGLTIEDLTDSFAPYLT|MAEGLKLAALTFDKDVSKLSCCA
FI.KLV.IEEGSHRLI |GVQAVAPEAGELIQT.AALAIRNRMTVQELADQLFPYLT|MVEGLKLAAQTFNKDVKQLSCCA
FI.KLV.VEEGSGRLI |GVQAVAPEAGELIQT.AALAIRNRMTVQELADQLFPYLT|MVEGLKLAAQTFNKDVKQLSCCA

F1-Help  F2-Bar   F3-Lock   F4-Select   F5-Dot         F10-Commands
```

Figure 6.4: The 'A' has moved to the right, bringing the green hydrophobic residues into alignment. Other residues have moved as a result.

In "Medal" menus of commands and the on line explanatory 'help' information are combined in one system. When selecting commands the lower third of the screen contains a menu of commands. The command currently selected off the menu has help information about it displayed in the top two thirds of the screen (figure 6.2).

"Medal" uses an initialisation file which records path names. These paths indicate directories in which sequence and alignment files are stored and the name of the alignment most recently worked on. When "Medal" is invoked the most recent alignment is fetched into memory so that alignment editing can resume where it was last left off.

Platforms and interfaces

The editor interface design illustrates the influence of the platform. On a machine with a slow transfer between screen and memory, update of help information as each menu command was selected would have lead to a very slow response and would have been impractical. Multiple sequence editors on other machines follow interface practices for those machines. The "Lineup" editor uses interface features from the VAX/VMS editor "EDT" such as the use of ctrl Z to end screen based input. The Unix "Mase" sequence editor uses binding of keys to longer commands similar to that found in the Unix editor "emacs".

Sequence editing features

Other features of "Medal" were designed specifically to assist multiple sequence alignment editing. Normally in "Medal" all sequence characters are shown in grey. Pressing a key on the keyboard causes all occurrences of the character to be displayed in a preselected colour. Residues which are conservative substitutions use the same colour. Pressing the key again causes all occurrences of that character to revert to grey. This feature helps in locating by eye patterns of similar residues.

"Medal" allows regions of the sequence to be delimited. A vertical bar in an alignment prevents changes made on one side of the bar from affecting in Chapter 2. Storage for the full path matrix is not necessary if only scores are required. In the searching phase only two columns at a time are needed. For reconstructing the alignment, the path matrix size can be a problem even on mainframe machines. In the GCG implementation path matrices are limited to 1,000,000 elements. This is sufficient for two sequences of length 1000. On microcomputers the memory problem is more severe.

The "Prosrch" program described in Chapter 4 has one solution to this problem. With the "Prosrch" program both endpoints of any alignment path are obtained in the searching pass. Whilst this adds a substantial overhead to the searching time it simplifies the post processing[1]. When both endpoint positions for a Type III alignment are available reconstruction of alignment is equivalent to a Type I alignment. For Type I alignments this reconstruction can be done in a space efficient manner using 'divide and conquer' techniques (Hirschberg, 1975). These require recalculation of some of the path matrix entries.

"Prosrch" in fact uses a more sophisticated hierarchical approach. It tries successively more complex algorithms in turn. This sequence is optimised for alignments with few gaps (J.F. Collins, ICMB Edinburgh, personal communication) and is extremely efficient in these cases.

The new serial Type III implementation described in this chapter finds the endpoint but does not find the starting point of an alignment. However, as each column of the path matrix is calculated, the software finds the score and location of the best path ending in the column. These values are used in finding the score for the overall best path and the location of its endpoint. The requirement is for an efficient way to reconstruct the alignment given only this information for each column.

[1]To do this, each path matrix element must carry start coordinates of a path in addition to a score.

Method:

The strategy employed to do this, like the divide and conquer approach, uses recalculation to reduce memory requirements. The strategy is not guaranteed to use as few recalculations but its behaviour is good in practice. The method uses the stored values for the best path ending in each column to reduce the likelihood of needing recalculation. Many of the recorded locations are actually on the best path. The reconstruction is particularly rapid where this is the case. Reconstruction proceeds as described in Chapter 1 except that only ten columns of the path matrix are available at a time. Moreover the values present may be lower than they should be. Consequently the retrace procedure may be unable to continue before having retraced the full path. In that case, path matrix recalculation is started from fifteen columns prior and generates the ten columns immediately before the column of the current cell. This recalculation starts by setting the first of the fifteen column's scores to zero, whereas in fact some of the values should normally be positive. This is the reason for some values being lower than they should be. The recorded best endpoints in a column are put in place after each column is calculated. This step ensures that if any one of the fifteen recorded best endpoints prior to the current cell is actually used on the best path, then the path being reconstructed can be continued. This is usually the case for high scoring alignments. The approach also works if the path started within 15 steps of the cell currently reached. This tends to be the case for short low scoring alignments. If, however, this stage also fails to extend the path, recalculation can be started from the start of the sequence and will yield correct scores, rather than reduced scores, for the immediately prior ten columns.

Discussion:

This strategy works well in practice. It also is simpler to program than the 'divide and conquer' technique. This results in a shorter program. The divide and conquer method needs to change the direction in which sections of the path matrix are calculated. The new method does not. It uses the same score calculation code for reconstruction as used in the search. Since this code has been heavily optimised, reconstruction of alignments is rapid. The method also extends gracefully if more memory is available. Storage for more columns of the matrix leads to lower likelihoods of repeated recalculation.

Database compression (Disk space)

Problem:

A second problem with microcomputers is limited disk space. One investigation concerned methods to compress the sequence database. The method developed has an exceptionally rapid decompression algorithm.

Compression technique:

Standard compression algorithms, such as the Lempel-Ziv algorithm, work by identifying short patterns which occur many times and choosing compact representations for these patterns (Welch, 1984). The new method recognized a common kind of repetition that occurs in sequence databases - longer sequences of characters that are repeated just a few times.

`0vvvvvvv`	Single character.
`1nnnaaaa aaaaaaaa`	Repeated run of length 3-9.
`1111aaaa aaaaaaaa rrrrrrrr`	Repeated run of length up to 256.
`vvvvvvvv`	Character value (7 bits).
`nnn`	Run length in range 3-9, represented by 000 to 110.
`aaaa aaaaaaaa`	Offset to previous occurrence of run.
`rrrrrrrr`	Run length in range 0-255.

Figure 5.1: Coding scheme used to represent repeated runs. Above: The three forms in which data is represented in the compressed file in terms of bit fields. Below: Interpretation of the bit fields.

A compressed run is represented by a word (two bytes) with the top bit set. The lower twelve bits represent the relative location of the run. The remaining 3 bits represent the length of the run. 000 to 110 represent runs of length 3 to

9. Longer runs are represented by 111 and the byte after the word gives the actual length up to 255 characters.

The standard compression methods would be unable to exploit such longer patterns nearly so well. The Lempel-Ziv compression algorithm, for example, needs to 'learn' each shorter run in the long repeated run before it can learn the long run and represent it with maximum efficiency. The effect of this is that to 'learn' a longer run, the Lempel-Ziv algorithm must see it at least as many times as its length. In the database, many of the long repeated patterns occur only a few times. With the new method a long run only needs to be seen once for future occurrences to be compressed.

Using the algorithm 2.5Mb of the PIR 23 database was compressed to 1.2Mb. Later, commercial software for compression, the program "PKZip" (PKWare Inc, WI 53217, U.S.A.), was available. This algorithm achieves the same compression factor, though it is not clear what compression algorithm it uses.

Discussion:

For machines for which disk performance is a severe problem, compression gives an effective increase to the capacity of the disk. It also gives an effective increase to the speed of disk read access since decompression is more rapid than disk to memory transfer. Compression software improves the effective performance of the disks. In practice the method was only used to reduce the number of disks needed for data redistribution.

Data compression may become more important as the 'human genome project', an international project to sequence the $3x10^9$ bases of the human genome develops (Gordon, 1988). When distributing data to a large number of users, the potential savings from reducing media costs could conceivably be significant. The increased speed of access is unlikely to be a critical factor as for sensitive searching on low cost hardware the rate limiting step is the actual comparison.

The technique is particularly attractive for representing slight variants of a gene. At the moment there is an inconsistent policy over representation of sequence variants in the database. Sometimes a variant sequence is given a new sequence entry. At other times it is recorded in feature tables that give alternatives at particular sites. Variants so represented are not searched by current software. Using the kind of compression suggested here, full entries could be made for these variants with minimal media costs.

Tripeptide matching (Speed).

Problem:

For part of the study a very fast approximate sequence comparison routine was required (Chapter 7). A coarse method was acceptable as only the very strongest similarities needed to be found.

Method:

The coarse comparisons were made using a program to count exactly matching tripeptide words shared by pairs of proteins. The program took each protein in turn and used it as a query against the database. To make the counting of shared tripeptides rapid a tripeptide index for the query sequence was used. To increase the efficiency of disk access, software to cache data from the database was written.

A near eight fold speed improvement on this basic method was subsequently achieved by searching with eight query proteins simultaneously. This reduced the disk overheads by a factor of eight and, by splitting integer scores into fields, one addition operation was made to serve the update of all eight proteins scores. Overflow of the fields was avoided by periodically using the fields to update scores with a wider range.

With these enhancements the comparison program could perform 32,000,000 coarse tripeptide based pairwise sequence comparisons over a weekend.

Discussion:

Even more rapid techniques are possible using sorting of subsequences from the database. Here a technique that compared each protein pair in turn was required partly to test the software framework for total database searching. A relatively simple method was found to be satisfactory.

Annotation browser (Portability)

Problem:

A screen based application for examining lists of results from database searches was written. The program provided a rapid method for cross referencing between search results and sequence annotation databases. The first version was written for emas, a mainframe computer which had ample disk memory and which held the sequence annotation files. The program, which was called "Xref", was initially written in IMP, the language of choice on the emas computer. Later the software was required for other machines. Since the program was not computationally intensive, portability seemed to be an important design goal for the rewrite.

Methods:

"Xref" was rewritten in Turbo Pascal for the IBM microcomputer with the intention of porting it onto a Unix machine. This was initially thought to be a good development route. As well as producing a version of the software for the microcomputer, it gave the opportunity to use the excellent development environment of Turbo Pascal in rewriting the code.

Documentation on the Unix Pascal that was available for the Unix machine turned out to be insufficient. There was insufficient information about file mapping, control of the intermediate PAD I/O computers and access to terminal characteristics. In addition the Unix Pascal compiler did not support some of the Turbo Pascal type casting operations that were essential to the program's operation. Just as IMP is the best supported language on the emas computer, so C is the preferred language for Unix machines. More facilities were readily available in C and C seemed to offer possibilities for a more portable implementation. The program was therefore rewritten in C using the Unix terminal independent screen and cursor control subroutines 'Curses' since one factor which reduces portability is having software depend on specific hardware features. The 'Curses' routines are aptly named. Using the routines an addition of a single line at the top of the screen resulted in the whole screen being redrawn to achieve reverse scrolling. This was slow and made the program useless as an interactive tool. The compromise solution adopted avoided the 'Curses' software and provided direct cursor control sequences for VT100 compatible machines (Wyse, 1984). These sequences gave correct scrolling action for Sun workstations, VT240 and Wyse 75 terminals and microcomputer terminal emulators. In practice this restriction in terminal type did not cause problems.

Discussion:

The observation from experience with "Xref" was that translation of software to different languages provided a rapid route to porting software between different machines. Overcoming the portability problems of the Pascal version would certainly have taken longer than rewriting in 'C'. Although the program was not compute intensive it seemed to require a compromise in portability. This was a result of the need for fast screen I/O.

Optimisation to "Prosrch"

Problem:

The PC program demonstrates the possibility of sensitive database searching at reasonable speed using the Type III algorithm on a microcomputer. The software can be used to do a comprehensive comparison of every pair of sequences in the database (Chapter 7). However this takes several weeks of computer time. A natural question to ask is: "Can the same techniques also be applied to accelerate database searching on a parallel computer?". If so, comprehensive sensitive pairwise comparisons of databases could be made on a routine basis.

Methods:

To investigate the possible optimisations of a parallel program, source code for the "Prosrch" program was examined and modified in the time critical sections. These modifications illustrate the potential for optimisation but to fully evaluate them would require substantial changes elsewhere in the software.

Experience from optimising the serial implementation drew attention to aspects of the calculation where savings could be made. The programming techniques to do this were different; none of the techniques discussed for the serial code apply. The details of the methods are peculiar to the parallel computer's architecture and the "Prosrch" program and are described in Appendix 4.

Discussion:

The serial techniques used to achieve speed on the microcomputer are not applicable to the parallel computer. The serial techniques rely on case analysis to make processing more rapid. On the parallel machine many cells are processed at once. These must be processed with the same set of instructions. On the parallel machine separate processing of different cases slows down operations. Multiple cases take the sum of the time for the different cases, rather than a weighted average of the times. Radically different optimisation techniques need to be used.

The study provided an alternative set of optimisations suitable for the parallel machine. More important than a potential for an estimated eight fold speed improvement was a dramatic reduction in code size arising from the optimisation method. This should aid maintainability and future modification of the software for other uses. The techniques described in Appendix 4 would also be appropriate for efficient implementation of the more computationally expensive string comparison algorithms of Chapter 9.

Concluding remarks

Practical considerations draw attention to computational problems that are different in kind to purely theoretical studies. In the main these can be tackled by adapting and applying combinations of standard computing techniques. For practical systems, it is not so much individual techniques as combinations of techniques that matter. This is so both at the level of individual subroutines, such as the new Type III subroutine, and at the level of applications programs.

Chapter 6: Multiple Sequence Alignment

Multiple sequence alignments show in a compact format relationships between several protein sequences. They differ from pairwise alignments by having extra rows for additional related sequences in a family. The presentation of information is more condensed than if a set of pairwise alignments were shown. Strong patterns present in all sequences are readily distinguished from patterns present in only some sequences. These patterns would be harder to see by examining pairwise alignments. An example of a multiple alignment is shown below:

```
        ++++++    + ++.  + +.+  ..     + + + + +++ ++++      .+  .
A30007 :APKIFGGEIKTHILLFLPKSVSDYDGKLSNLKKAADGFKGK ILFVFIDSDHTDNQR
A26289 :APKIFGGEIKTHILLFLPKSVSDYEGKLSNFKKAAESFKGK ILFIFIDSDHTDNQR
ISRTSS :APKIFGGEIKTHILLFLPKSVSDYDGKLSNFKKAAEGFKGK ILFIFIDSDHTDNQR
R3EC2  :KPRIFGARNKVHIIN LEKTVPMFNEALAELNKIA SRKGK ILFVGTKRAASEAVK
R3NT2  :APYISAKRKGIHITN LTRTARFLSEA CDLVFDAASR GKQFLIVGTKNKAADSVE
R3LV2  :APYIFTERKGIHIIN LTQTARFLSEA CDLVANASS KGKQFLIVGTKYQAADLIE
```

Identities '+' and conservative substitutions '.' are marked where four or more of the residues are identical or conservative substitutions.

The top three sequences are strongly related to each other, so too are the lower three. The relationship between the two families is less certain, the similarity between sequences R3EC2 and A30007 providing most evidence for there being a valid link. This particular alignment was produce to help investigation of a hypothesised link between protein disulphide isomerases, top three sequences, and ribosomal sequences, lower three sequences (see Chapter 8). Multiple alignments present more detailed information about the interrelationships between sequences than a pairwise alignment can. Individual columns of a multiple alignment show the range of variation at a site, assuming that is, that the multiple alignment correctly aligns residues that are in structurally equivalent positions.

residues on the other side. This feature is also present in "Mase" and "Esee".

The editor "Alma" allows groups of sequences to be locked together. Insertions or deletions in any one of these grouped sequences affects all sequences in the group. In "Medal" locking together is done on a per residue basis. Such residues are shown with a coloured background. Residues which are locked together stay aligned whatever other changes are made. Residues which are locked together move together whenever any shift, insertion or deletion moves one of them. This system makes it possible for the sequences locked together to vary from column to column. This approach is more general than whole sequence locking. Photos in figures 6.3 and 6.4 shows residue locks in action. The lock on residues can be set and removed easily by a single keypress. Also an automatic scan can be made that puts in locks for all currently aligned residues of similar type. Locks are also placed after automatic alignment.

"Mase" and "Homed" have more limited whole sequence locking than "Alma". They are restricted to locking together groups that consist of all but one of the sequences.

Assessment of editor features

The editor part of "Medal" demonstrates the advantages of using computers of the PC kind rather than a mainframe for multiple sequence editing work. In many ways the advantages parallel those of using a PC word processor over a mainframe text editor, a faster screen update leading to more information on the screen at one time and user friendly help and menu facilities.

Additionally the "Medal" editor introduces a new concepts to multiple sequence editing; the locking together of sequences on a per-residue basis. The features in "Medal" were largely influenced by use of the program in creating and modifying multiple alignments. Using per-residue locks on their own was found to be not entirely satisfactory as for larger alignments changes can involve setting and resetting many such locks.

One option in "Medal" combined automatic placement and removal of bars at the boundaries of regions of sequence conservation with locking together of the similar sequences over the region of conservation. This was found to be confusing to other users of the software. For the kinds of editing for which this option was designed an alternative would be more useful. This would give the user temporary locking together of any subset of sequences over a region delimited by the bars. Existing residue locks would still be active. Were there time to do so, this alternative option would be added in place of the present option.

Sizes

Of the editors considered, "Medal" has the smallest capacity for sequences.

	Max. number of sequences	Max. sequence length
"Lineup"	31	100,000+
"Homed"	50	10,240
"Mase"	100	10,000
"Esee"	21*	18,000*
"Alma"	300	60,000
"Medal"	21*	6,000*

*Figure 6.1: Sequence capacities of various multiple sequence alignment editors. Figures marked * are for programs on microcomputers. For microcomputer programs the maximum size and maximum number of sequences are not available simultaneously.*

Additional work would be needed to raise the number of sequences and increase the maximum sequence length. Data compression techniques, as described in Chapter 5, could be one way to achieve this. The alternative is to make greater use of disk memory. Much larger datasets could be accommodated by holding parts of the alignment not currently being displayed in disk memory. Space could also be saved by making the automatic alignment process, currently part of the "Medal" program, into a separate program.

Automatic alignment methods

Ideally an algorithm for automatic multiple alignment should find patterns which do not show up when only pairs of sequences are compared. One approach to this is a multidimensional extension of the Type I algorithm (Murata *et al.* 1985). Instead of a two dimensional path matrix, a K dimensional array, where K is the number of sequences being aligned, is used. Each additional sequence of length three hundred increases the workload three hundred fold. Whilst cutting corners in the match matrix, that is calculating only elements near the main diagonal, dramatically improves this situation, it brings with it a risk of missing similarities when large insertions or deletions are required. Because of large memory and time requirements, this direct approach has been limited to three sequences.

Subsequent to Murata *et al.*'s work, a more sophisticated method for restricting the volume explored in the multidimensional path matrix has been developed (Lipman *et al.* 1989). The method uses an alternative formulation of matching in terms of difference scores rather than similarity scores. The method extends the multidimensional method to up to six sequences. Unlike conventional corner cutting, the alignments this method finds are guaranteed to be optimally scoring by the scoring scheme.

Close examination of the method suggests that the computational costs grow very rapidly with increase in sequence dissimilarity, even where this is confined to short local regions. Thus, the method is applicable to up to six sequences only when the sequences have very high levels of similarity.

A problem which is noted in the paper is that the scoring strategy needs adjustment where one subfamily is heavily represented in the set, otherwise the alignment is determined by these sequences, being then an alignment to the consensus for this subfamily. This adjustment involves a manual intervention to identify clustering of related sequences.

Using exact matching

Some algorithms overcome the problem of computational demands by using exactly matching words (Sobel & Martinez, 1986; Bains, 1986; Santibanez & Rhode, 1987). These algorithms can be seen as multidimensional extensions of the Wilbur & Lipman (1983) approach. These word based alignment methods have the same advantages as the word based pairwise methods - simplicity and ease of calculation. The methods suffer from the same problems as the pairwise exact word based methods. That is, lack of sensitivity over amino acids of similar properties, inability to cope with single mismatches in a region of good matching and inability to deal with insertions.

A promising variant of the exact matching word methods removes the restriction that the amino acids be contiguous (Roberts, 1990). The variant, as currently implemented, only detects the interrupted similarities, the multiple alignment being performed manually. Allowing broken similarities overcomes the problem of single mismatching residues ruining an otherwise matching word and preventing its detection. This idea is examined again in the addendum in the context of database searching.

Alignment refinement

A technique used as an additional stage in a number of multiple alignment strategies is called refinement (Bains, 1986; Barton & Sternberg, 1987; Hennecke, 1989). After the initial multiple alignment has been made, individual sequences are removed one at a time and realigned against the remaining sequences. The process stops after a preset number of cycles or when no further improvement in alignment score is achieved. The technique will not correct very poorly aligned sequences but can fix 'glitches' where one sequence is obviously out of alignment relative to the rest. A modified form of refinement, claimed to have some advantages over the simpler form, removes and realigns groups of sequences (Subbiah & Harrison, 1989).

Refinement is popular partly because it is a simple addition to a program that already performs alignment of sequences to aligned sequences. A disadvantage of refinement is that realignment is performed against a consensus, so the criticisms of comparisons using consensus given earlier in this chapter apply. Refinement, as its name suggests, can improve patterns already detected by the initial method, but it is unlikely to detect patterns that the initial method does not find. If a particular shared pattern is not evident from the consensus, for example if the shared pattern is found in only two of the sequences, refinement does not help. If the initial method finds such pattern, refinement is likely to lose the pattern again unless the weaker pattern is bounded by strongly conserved patterns that do show in the consensus. Refinement was not used in the alignment algorithm of "Medal".

Multiple alignment based on pairwise alignment

One of the most straightforward multiple alignment methods is based on pairwise alignments. The method is due to Taylor. He compared results of this method to those of multidimensional extensions of the NWS algorithms as follows:

> "Reasonable multiple sequence alignment can be achieved by a simple method. The results are equivalent to those obtained using a complex algorithm that considers the sequences simultaneously during alignment." (Taylor, 1987b)

Taylor built his multiple alignment using selected pairwise alignments. The selection process required only a few pairwise comparisons. The method considered addition of a new sequence to a multiple alignment of a few sequences by pairing either to the first or last sequence. Results of this approach depend on the order in which sequences are presented to the algorithm. This strategy seems most appropriate for cases where the sequences represent various intermediates between two extremes and are presented to the algorithm in an order that reflects this.

The approach used by "Medal" adds one sequence at a time to a multiple alignment, each time adding the new sequence most closely related to any sequences in the alignment and aligning on this pair. This approach seems appropriate to more arbitrary relationships between sequences than Taylor's and includes Taylor's pattern of relationships as a special case. Moreover this approach makes the alignment essentially independent of the order in which sequences are presented to the algorithm.

Consensus alignment

Closely related techniques to "Medal's" are used in two other methods of multiple alignment (Barton & Sternberg, 1987; Higgins & Sharp, 1989). Both these methods perform all pairwise comparisons of the sequences to establish the order of alignment. This frees the algorithms from dependence on the order in which sequences are presented to them. For reasons of speed the programs use the Wilbur-Lipman pairwise approach to perform all comparisons. The Type I algorithm is then used in actual alignment.

The program of Barton and Sternberg differs from "Medal" in aligning each new sequence to a consensus for the sequences aligned so far. This may give better results if the sequence being added is more similar to the consensus than it is to any of the sequences in the alignment. This consensus strategy for forming multiple alignments is inappropriate where the sequences show differing degrees of pairwise similarity. In these cases the 'average sequence' is likely to be much less similar to a given sequence than the most similar one of a set. This situation frequently arises in practice. Sequences being aligned are often obtained from closely and from more distantly related organisms.

Higgins and Sharp's "Clustal" program also uses consensuses. It progressively aligns initially separated subfamilies of sequences. It combines alignments by aligning pairs of consensus patterns. The problems of consensus sequence alignment are less in this case. Because of the order in which alignments are made, most patterns present only in subfamilies can be found before the subfamilies are combined. In particular, anomalous patterns present in just two of the sequences can be found. Anomalous shared patterns seen in examining alignment by hand were nearly always present within subfamily groups. Consequently "Medal" and "Clustal" should have very similar behaviour in most cases. In exceptional cases such as the multiple alignment at the start of this chapter, the link between the families is clearest from one pair of sequences. Only in such cases would "Medal's" approach be expected to give superior results.

Equally there will be special cases where a clearer picture of similarity arises from "Clustal's" approach.

Examining the different methods, we see that similar principles underlie several different approaches. "Medal's" method can be seen as combining specific elements of Taylor's and Barton and Sternberg's techniques to give similar advantages to the "Clustal" method of Higgins and Sharp. In fact the relationship of "Medal's" algorithm to "Clustal's" is closer than initially appears. In "Medal", introducing sequences one sequence at a time is an algorithmic convenience. The alignment "Medal" produces would be unchanged were it instead produced by combining subfamily alignments basing alignment of these alignments on the most similar pair of sequences in the two subfamilies.

Use of the Type III method in "Medal"

One motivation for developing "Medal" was to test the utility of the new Type III software in practice. To perform the comparisons for clustering rapidly, "Clustal" uses an approximate word based method. "Medal" is able to use the full Type III algorithm, and performs the calculations faster as a result of the optimisations to the implementation. The advantages of this in practice are slight. The reason for performing these comparisons at all is to distinguish subfamilies from each other. For this purpose, an approximate comparison method is as satisfactory as the more extensive method.

In performing the pairwise alignments for the multiple alignment, "Medal's" Type III alignment has some advantages over the Type I alignment used in other programs. For closely related sequences the Type III 'best local homology algorithm' aligns whole sequences and there is little difference between using Type I and Type III alignment. For strongly and for less strongly related sequences, the Type I algorithm forces alignment from end to end. Improvements in the matching in unrelated regions may take precedence over the alignment of related regions. In particular, an unmodified Type I alignment incurs penalties for unmatched residues at ends of sequences and attempts to make the starts and ends of sequences correspond. This problem has been addressed by modification of the Type I algorithm to suppress penalties for the gaps at the ends of sequences (Devereux *et al.*, 1989b). This modification allows one sequence to terminate before the other without incurring a penalty. The local algorithm copes with this problem without any modification. For Type III alignment, unmatched residues at the ends of sequences lie outside the local region of alignment and incur no penalty. As well as the case where one sequence is a truncated version of the other, Type III alignment can also handle sequences that differ at their extremities, a case which the modified Type I algorithm does not handle well. Experience of multiple alignments made by hand indicates greater variability in terminal sequences, so the ability of Type III alignment to handle this case is important.

Recursive local alignment

A disadvantage in using the unmodified local method is that only one local matching region will be found even if there are several. Additional local matching regions are of interest to the biologist. Those regions compatible with regions already found could be used to make a more complete alignment of the two sequences.

The additional regions of similarity can be found by reanalysing the parts of the sequence not included in the strongest local alignment. A recursive sequence alignment method was developed following this principle. It was used in "Medal" in place of the simpler Type III algorithm. The procedure finds the best local region between two sequences, aligns these parts of the sequences and then aligns the initial and terminal unaligned regions of the two sequences by calling itself recursively. The recursion stops when the alignment score for two sequence segments drops below a preset threshold chosen to correspond to insignificant alignment, the value 70 being found suitable in practice. The method is an advance on the Type I and Type III methods. It includes all the information yielded by the Type III method because the Type III aligned region is one part of the alignment formed by the recursive method. The method maintains the advantage of the Type I algorithm that separated regions of matching are found.

A way to view the new pairwise method is that it handles two levels of matching. It handles good matching in the local regions it aligns and poor matching in the regions between. There is a natural biological rationale for doing this. Over regions in the core of the protein, or in active sites in enzymes, a high level of conservation of residues is expected. Changes in these locations will be strongly selected against. Changes in the core are likely to radically change the folding of the protein and changes in the active sites will change the activity. Loops on the protein surface, on the other hand, are in general less constrained and more likely to change. They are expected to be more tolerant of point mutations and of changes in length. Structural considerations lead one to expect a variation in variability along the length of a sequence. As discussed earlier in this chapter, examining variation in variability is one reason for constructing multiple alignments. The methods used in comparing sequences should take account of such variation.

Comments on alignment methods

Multiple sequence alignment algorithms are an active area of current research. As with pairwise comparison, nearly all multiple sequence alignment methods produce reasonable answers when the similarities are strong and poorer results with weaker similarities. Given the current state of development of automatic multiple sequence alignment software, a combination of automatic and manual methods is, and is likely to continue to be for the near future, the best method for aligning weakly related sequences. Experience from this can then help guide the design of new algorithms. For weakly related sequences, "Medal's" automatic method produces reasonable alignments. These are best regarded as starting alignments for refinement by hand.

Use of the new computer tools

The software for producing multiple alignments was used to examine around 50 sequence families. This work was predominantly with sequences grouped by the total database comparison program (Chapter 7) and included some of the most tenuous relationships. The main observation of importance was the already remarked on frequent presence of islands of good matching separated by regions of poor matching. It is this which lead to the recursive alignment strategy. In the sample alignment at the start of this chapter the island patterns 'APKIF', 'KTHIL' and 'KGKILFV' show strong matching. The regions between have a low level of similarity. Other observations concerned changes in sequence length and repeated subsequences.

Insertions

A frequent occurrence in sequence families is of inserted sequence in one or a few of the sequences. The top two sequence fragments shown in the alignment below are from different dihydrolipoamide dehydrogenases and the lower two from glutathione reductase and mercuric (III) reductase:

```
RDHUU       : TAP..HIL......IATGGMP
DHDL$YEAST  : TVKEDHILDVKNIIVATGSEV
RDPSHA      : VVMFDRCL......VATGASP
RDEBHA      : VVAFDRCL......IATGASP
```

Direct repeat

In this example from the AIDS HTLV-III virus coat protein, the inserted sequence is an exact repeat of prior sequence. The coat protein has a number of known highly variable repeat regions of which this is one (Stavich *et al.*, 1986). In isolate BH10 the exact repeat of 'FNSTW' corresponds to an exact repeat at the DNA level.

```
BH10 : CNSTQLFNSTWFNSTWSTKGSNNTEGSD
ARV2 : CNTTQLFNNTW          RLNHTEGTK
HAT3 : CNTTQLFNSTWN     STEGSNNTGGND
```

Interestingly in BH10, upstream of the repeat, the sequence 'NST' that occurs in the insert 'FNSTW' is found once again. The repeated 'VK' in the yeast sequence in the previous multiple alignment might conceivably be a remnant of a duplication of this kind.

For the AIDS virus, a finer repeat which does not show at the amino acid level can be hypothesised. The threefold DNA repeat **'aactc aactc aactc'** could have given rise to the sequence 'NSTQL'. The actual DNA sequence has the pattern **'aattc aacac aagtg'** and the weaker repetition is not strong enough to support the hypothesis that finer scale repetitions are related to longer scale ones.

Repeat in initial sequence

In many examples of sequences that were compared by multiple alignment it was found that the proteins shared a region of strong similarity and showed most dissimilarity at their termini. Towards the ends of the proteins lower levels of amino acid matching were frequently accompanied by length changes.

This example and the next example come from a family of ribonucleotide reductase small subunits. Two of these sequences were about 100 residues longer than other small subunit proteins considered. The alignment shown below is within the initial additional residues of the two sequences:

```
         ****  .  *    .**.
MUSSU  : SKAARRIFQDSAELESKA
YSTSSU : SKAAADALSDLEIKDSKS
```

The sequence similarity shown here is far weaker than in the main part of the alignment. Moreover, the pattern shown occurs at different positions in the initial sequences. In MUSSU the pattern is 60 residues further from the start of the protein than it is in YSTSSU. This pattern is not found by the Type I pairwise alignment algorithm nor by unmodified Type III alignment. For both these algorithms the penalty for the large gap of 60 residues more than outweighs the potential to increase score. Nor, unfortunately, does the modified Type III algorithm find this region since the match scores below the preset threshold at which recursion stops. In fact, this region of similarity was found by eye, using the editor to move the sequences relative to each other to bring short patterns into alignment.

For these two sequences there is some evidence that the initial sequence has biases in sequence not present in the main sequence. For example, elsewhere in the YSTSSU initial sequence, the tetrapeptidess 'SKDA' and 'ELET' occur. These show similarity to the sequences 'SKAA' and 'ELES' in the region of the MUSSU sequence shown here. These repetitions in the leader sequences may be due either to mutational processes causing repetitions in the DNA or to a selective pressure for specific short patterns.

Pattern movement

This example illustrates how alignments are selective in the patterns of similarity which they can show. The pattern in EBVSSU at position one would line up better against HSVSSU and VZVSSU in position two than does the pattern actually in that position. However, this would disrupt the strong matching between position one and two, and the position shown is better. Nevertheless the possibility of an alternative position is of interest.

```
                1                  2
             C. NNY.            C. NNY.
             *  *  *****  ** *     **        *
EBVSSU     : CLANNYISRDELLHTRAASLLYNSMTAKADRP
HSVSSU     : CQSNDLISRDEAVHTTASCYIYNNYLGDHAKP
VZVSSU     : CQFNDLISRDEAIHTSASCCIYNNYVP..EKP
```

Possibly the two sites either side of the strongly conserved region of matching have similar functions.

Concluding remarks

Currently, for smaller sequence alignments, microcomputers seem to provide a better vehicle for multiple sequence editor software than mainframe computers. This is principally because microcomputers permit a more interactive user interface than mainframes do.

Use of the "Medal" program shaped the design of its user interface. Use of "Medal" in the study of many alignments motivated the development of interface software to simplify formation of a new alignment. Use by other researchers motivated provision of on line help information integrated with the command menus.

Experience in manual editing of alignments lead to the recursive local alignment method for pairwise alignment that aligns separated islands of good matching without forcing the entire sequences to align.

Examination and manual adjustment of many alignments drew attention to small repetitions in proteins associated with higher variability. Such features of sequences pose problems which methods for alignment must tackle. Possibly recognition of repetition within the comparison algorithm will be important to future automatic methods. Automatic recognition of correlations in sequence variability and repetition would also be of potential interest to biologists.

Chapter 7: Comprehensive Database Analysis

Organisation of sequence data

In the PIR database each protein sequence is annotated as belonging to a sequence superfamily, family and subfamily. These groupings perform an important function in organising the information. Organising the relationships between sequences is crucial to the understanding of biological sequence data.

New methods for organising the sequence data are being developed. Multiple sequence alignments, as examined in the previous chapter, are central to the proposed methods:

> "Because of the importance of alignments to the study of protein sequences, the organisation of the Protein Sequence Database is being redesigned so that its fundamental structure will include alignments of related sequences. The current superfamily organization will be replaced by one based on alignments, i.e., each alignment will define a set of related sequences or subsequences." (Barker *et al.*, 1990)

The new organisation of data will include information about shared patterns in the sequences. The 'Prosite' motif directory (Bairoch, 1989a) discussed in Chapter 4, represents one step in this direction. It relied on published knowledge about families and shared pattern in proteins.

A complementary approach is comprehensive computer analysis of the databases to automatically organise the data. Comprehensive sensitive comparison may be able to uncover previously overlooked patterns of similarity. The task of comparing all pairs of proteins in a database is several thousand times more computationally demanding than the comparison of a single sequence against a database discussed in Chapter 4.

Comprehensive pairwise comparison

To perform large numbers of sensitive protein sequence comparisons rapidly one group has implemented the Type III algorithm on an exceptionally powerful computer (Jones *et al.*, 1990). The Connection Machine (Thinking Machine Corporation, Cambridge, MA. 02142-1214), a supercomputer with a cost of several million dollars, was used to perform comprehensive comparison of all pairs of proteins in a test database of 200 proteins. With this software Jones *et al.* have investigated the use of different parameters in searching. This work is continuing. They are also currently working on scaling the search up to perform 50,000,000 sequence comparisons for a less restricted database.

Methods and data used in comprehensive search

The approach adopted in this work has in common with Jones *et al.*'s approach use of the Type III algorithm and the comparison of all sequence pairs in a database. Rather than using a supercomputer, the new rapid implementation of the Type III algorithm was used. This made it feasible to do the analysis on a microcomputer. The Dayhoff 100 PAM matrix was used for scoring. Choice of this value was based on experience of single searches using "Prosrch" at the Edinburgh Biocomputing Research Unit. Experience shows that a choice of 100 PAMs and an indel penalty of 14, rather than the more usual 250 PAMs, gives better general performance (J.F. Collins, ICMB Edinburgh, personal communication).

An indel penalty of 10 rather than 14 was used. Whilst this made the alignment process less rapid, it had two advantages. Firstly, it exploited a key aspect of the Type III algorithm, the ability to accommodate gaps, to the full. Secondly, use of a higher indel penalty can only decrease the score for an alignment. If required, significant results for some higher indel penalty could be determined by re-examining only high scoring results obtained at the low indel penalty. To change indel penalty in the other direction would require recomputation of all comparisons.

In this work the 'PSeqip' 1987 compilation database containing versions of the protein databases Swiss-prot, EMBL and translations of open reading frames from the DNA database Genbank was used (Claverie & Sauvaget, 1985). The 'PSeqip' database contained 8117 proteins, a total of 2,500,000 residues. At the time the decision to use the 'PSeqip' database was made, many open reading frames from DNA sequences were not being included in the other protein databases.

Two factors make the database analysis that was performed less useful than would be desirable. Firstly, more up-to-date databases exist. Ideally, the analysis should be repeated with a more recent database. Using the older database, many of the unidentified genes which showed similarity to known genes may now have been identified. For an up-to-date database such similarities would have current relevance.

A second reason to repeat the analysis concerns a problem caused by memory limitations. This affected comparison of sequences longer than 1000 residues. These longer protein sequences were mostly viral polyproteins. They were only compared with sequences that were shorter than 1000 residues in length and prior to them in the database order. The comparisons of proteins shorter than 1000 residues were, however, comprehensive. This problem, a result of memory restrictions in the comparison algorithm, was subsequently removed but the additional comparisons have not been made.

Need for analysis methods

The production of scores for similarity between pairs of proteins solves just one part of the analysis problem. For a comprehensive search to be of biological use, methods for analysis of the results must be developed. Whether previously unsuspected similarities are found or not, an analysis of the results is vital to casting light on the value and limitations of the Type III algorithm and on the comprehensive comparison approach. A database of 8000 proteins generates 32,000,000 pairwise comparison scores. Methods were needed to winnow the comparison results to find unexpected sequence similarities likely to be of biological interest.

Coarse searching

Before performing the comprehensive set of comparisons it was decided to make a coarse rapid pairwise comparisons of proteins in the database (see Chapter 5 for computing methods). This data was to act as a reference set of known similarities.

The similarities found by this coarse method were of sufficiently marked strength that any reasonably sensitive method should find them. Examination of the collection of similarities confirmed that no previously unsuspected similarities were contained in it. Most of the strong similarities were between variants of proteins having the same function, for example, equivalent proteins from different organisms. Pairs of equivalent proteins exhibited extensive regions of high similarity, typically 70% amino acid identity or more. Other similarities found by the method were also already known. These included more local regions of high similarity in proteins which had virtually identical functional domains in common. This collection was now ready to act as a reference data set of known similarities.

Similarities found by the more sensitive search could be automatically checked against this set to exclude known similarities from further consideration. It was hoped that this would bring previously unsuspected similarities to light.

Actual use of coarse method

Generation of the coarse similarities data provided a rapid and thorough test for the framework to support comprehensive comparison. The framework included software to cache data from disk and to allow suspension and resumption of the search. The former enhanced speed, the latter was essential for extended running. It was estimated that the sensitive search would take two months to complete its run running on a 16Mhz IBM PC.

After the coarse search had been completed the new Type III algorithm was installed in its place. The reference collection of known similarities now acted as a convenient dataset for further development work on analysis methods. During the day, software for analysis of results was developed using the coarse dataset. During the night, the more sensitive sequence comparison algorithm was at work producing an improved dataset.

In fact, the reference dataset was never used in the manner originally intended. The development of analysis software and methods lead to an alternative method that organised the search results. This promised to be superior in bringing to light previously unsuspected similarities than using the coarse dataset to filter out known results. A program was written to implement this data reorganisation. The results of the comprehensive comparison were available after nearly fifty days of calculation.

Reduction of the similarity data

Some of the data reduction problems presented by a comprehensive database comparison can be illustrated by reconsidering how a biologist looks at the results of a single database search with one protein query sequence.

Visual inspection of an alignment in a list of results may show that it comes from a region of unusual composition. Biologists examining the list may choose to downgrade these similarities. They will pay less attention to similarities that are already known and well understood. They will mentally group results which are biologically related, matches of their query to sets of viral proteins or to receptor proteins. They will do this even where the matching proteins do not occur together in the database or in the results list. For routine searching this manual analysis is acceptable. The computer has selected a few tens of sequences that may repay closer examination from the database of thousands. With results of this kind for every sequence in the database, rather than for just one query, an alternative procedure is essential.

In particular, automatic ways to group results and to present information about links between these groups are needed. Use of the strategy for single searches would lead to an overwhelming task of manual analysis. Consideration of two natural ways to filter out less informative results shows up some of the specific problems in data reduction.

Reduction by threshold

One way to reduce the volume of results presented is to modify the single query method. First the results are sorted by score. Only the best of these results, those comparisons scoring above some selected threshold, are presented. When this method is applied to comprehensive comparison, larger families of related proteins dominate the output. These are similarities the biologist already knows about. Each family produces a number of high-scoring results proportional to the square of the family size. The haemoglobin family of around 430 proteins produces 80,000 high-scoring comparison results. None of these results come as any surprise. Unfortunately, a simple threshold must be set low enough to report them all, otherwise other biologically significant results will be lost. There is no question that the similarities within a known family exhibit evidence for biologically significant relationships. The problem is that the similarities are known already. Moreover any sequence showing similarity to a single member of a family is likely to show similarity to all members of the family. After the best of these similarities has been reported additional similarities to the other members of the family come as no surprise. The problem is the reporting of essentially the same results many times over.

Reduction by 'best choice only'

An alternative strategy to the threshold method reports only the best similarity for each protein. This produces substantially fewer results than the threshold method. With this method at most 430 results are reported for the haemoglobin-haemoglobin similarities, a 200 fold reduction. This approach gives a very drastic data reduction. Every myoglobin sequence is most closely related to other myoglobins. Every haemoglobin has its strongest similarity to another haemoglobin. The strategy would totally fail to show any link between myoglobins and haemoglobins, yet there is unquestionably a biologically significant sequence similarity between the two families. This similarity would have been detected in the search and would have been removed by the 'best choice' method of reduction. This method fails to find significant links between families.

Reduction using the reference set

As was originally intended, the reference dataset of similarities could have been used to define groups. Multiple similarities of sequences between these groups could then be reported once only. To a limited extent this would still result in multiple reports. These would arise when groups of sequences were similar by the criteria of the more sensitive search but were not similar enough to be grouped together on the basis of the crude search.

Tree based reduction

The reduction strategy developed for this work discarded a large number of links between proteins. It found a maximally scoring tree using the scores linking the proteins. Any link between two families that was kept was the best link between members of those two families. This follows from the tree being maximally scoring. Were a better scoring link between families available, replacement of the inferior link by the superior one would lead to a tree with a higher overall score. The tree property ensures data reduction. Any subset of n proteins have at most n-1 links between them in the tree.

The maximal scoring tree can be produced using a 'greedy' algorithm (Bollobas, 1979). Links are introduced one at a time between initially separate proteins. As links are added clusters aggregate. At each stage the highest scoring link which links two separate clusters is added.

None of the difficulties previously mentioned is a problem with this data reduction. For a family of 430 globins, 429 links are kept. On the other hand if the non-haemoglobin sequence that is most closely related to a haemoglobin sequence is a myoglobin, then the method guarantees that this link between haemoglobin and myoglobin families will be kept in the maximal scoring tree.

The method groups proteins together, but it does so simultaneously for each level of similarity. Because of this, information about groupings within each family is preserved as well as information about links between families.

Although not sufficient on its own, use of a threshold was important in the tree based reduction method. Using a threshold permitted an economy to be made in tree formation. Links with scores too low to give reasonable evidence for relatedness were discarded. In a search of this size scores below 100 readily arise from chance matching. Links scoring less than 100 were not included in the tree building.

Reducing the data by forming a maximal scoring tree is not a method suitable for forming phylogenetic trees that reflect evolutionary relationships. For such purposes methods of tree construction based on 'maximum parsimony' are popular. In this work we do not assert that the trees represent the historical process leading to the similarities. We require only a presentation of information about similarities in a condensed form. The maximal scoring tree does this. The maximum parsimony methods solve a different problem. Whilst the maximal scoring tree to link many thousands of proteins can be computed rapidly, rigorous maximum parsimony methods are by contrast impractical for more than ten proteins (Hein, 1989).

Tree formation

The construction method that was described for forming the maximal scoring tree required that individual links be introduced in order of score. A variation on the algorithm which forms the same tree introduces the links in any order. Each link that is introduced may complete a cycle of links. If so, the least scoring link in the cycle is removed. It can be shown that:

- Maximally scoring tree
- Sequential introduction of best link between clusters
- Addition of links followed by cycle breaking

all give the same tree. The third formulation is ideally suited to implementation on a machine with limited memory capacity such as the PC. This is the algorithm that was used. Sorting of the linking data prior to tree formation would have been problematic. The linking data, which had been written out to disk, took up 20Mb of disk space; considerably more space than was available in main memory.

One step in the variant algorithm was detection of cycles. Detection of the cycles potentially involved a time consuming operation. It might have been necessary to search most of the tree to detect each new cycle. This was avoided by working with a rooted tree. All links in the tree were given a direction. A 'rooted tree' has the property that following links sequentially always leads to the same root node. Initially the tree was set up with dummy links all pointing to one root node, each with a score of zero.

By using rooted trees only some nodes of the tree needed to be searched when a new link was added to link two nodes. The nodes examined lay on the sequential paths from the added link towards the root. These paths converged at some node, possibly at the root. As soon as a node common to both paths was found, a cycle had been detected. The least scoring link in the cycle was then removed. Removal of the least scoring link could require reversal of direction of some of the links in one of the paths in order to ensure that the tree stayed rooted. These searching and path reversal operations took time proportional to the length of the paths, which was in approximately logarithmic relation to the tree size.

The method presented here for coping with large sets of linking data and small available memory are minor adaptations of standard techniques in Computer Science. Whilst searching for relevant references for the techniques described here, the problem of tree formation from linking data held in external storage was found set as an exercise at the end of a chapter presenting tree algorithms (Aho, 1983). The hints with the exercise suggested addition of links followed by cycle breaking but not the method for detecting cycles.

Examining results

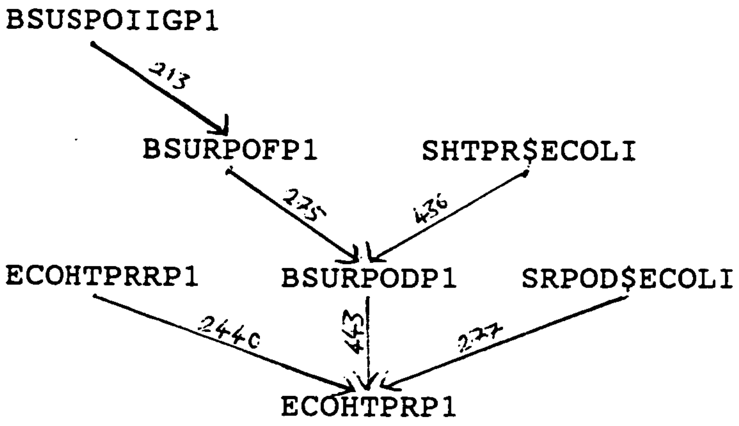
To examine the similarities represented by results held in the tree a program for 'browsing' the tree was written. This showed the alignment for any link and a dotplot for comparison of the two proteins connected by the link (see Appendix 1 for a discussion of dotplots). Choice of proteins was provided by direct selection or by movements up and down the tree hierarchy and between 'siblings' under a particular parent node. At any time the tree below a currently selected node could be displayed graphically.

The display of the branching structure of larger trees, whilst pretty, was of little use. The most useful aspect of the program was the combination of alignment and dotplot. This helped in checking the validity of links within the trees. Repetition and additional regions of similarity not found in locating the single best region for the alignment showed up particularly clearly on the dotplots.

The most useful method of all for examining the reduced trees was found to be printed output for small subtrees. This was non graphical. It consisted of lists of proteins within the subtrees, optionally with alignments. The threshold of 100 split the main tree into subtrees. These subtrees contained a mixture of weak and strong evidence for relatedness. A score above 200 provides very strong evidence for a relationship between two proteins. A threshold of 200 was used to further fragment the trees. Subtrees at the 200 threshold, that is subtrees in which all links were above 200 in score, represented strong family groups. The links scoring between 100 and 200 also formed a collection of subtrees. These represented 'linking data', the links between families. In both sets most of these subtrees contained twenty or fewer sequences. For small trees such as these, listing the sequence names, the links and the scores gave a clear picture of the relationships.

```
--Link--    Score Code name     Full name (and species)
1117->1108:  213 BSUSPOIIGP1   Sporulation protein. (B.subtilis)
1108->1107:  275 BSURPOFP1     37 KD minor sigma factor. (B.subtilis)
6978->1428:  277 SRPOD$ECOLI   DNA-directed RNA polymerase sigma chain. (E.coli)
5444->1107:  436 SHTPR$ECOLI   Heat shock regulatory protein. (E.coli)
1107->1428:  443 BSURPODP1     RNA polymerase sigma-43 factor. (B.subtilis)
1429->1428: 2440 ECOHTPRRP1    F33.4 heat shock regulatory protein. (E.coli)
1428->    0:     ECOHTPRP1     Heat shock regulatory protein. (E.coli)
--------------------Family 674 of 813--------------------------------------
```

Arranged as a tree:



Printed output was made of the families, the subdivisions and the linking data. A cross reference index was printed so that any protein in the lists could readily be found. The number of subtrees for the 200+ and 100-200 collections are shown below:

Strong similarities: Score 200+	
813 family groups	6221 proteins
Weaker similarities: Score 100-200	
720 linking families	2145 proteins

Note that some proteins occurred in both collections.

The tree construction method did not use prior assumptions about what protein families were in the database. It used instead the evidence given by the scores. The method of subdivision kept this information. Scores within the subtrees and scores linking the subtrees were presented. The subdivisions were simply a convenient way of presenting the tree data. All of the links in the tree were shown.

Larger protein families - problems.

Larger subtrees, these being exclusively in the 200+ collection, were dealt with by increasing the threshold once again. Increasing the threshold split these subtrees into smaller trees and produced additional sets of linking data.

One of the largest family groups contained 619 proteins. Nearly all of these were immunoglobulins. Raising the threshold to 400 split the family into fifteen smaller families separating, for example, gamma and other heavy chain immunoglobulins from lambda and kappa chain. This pulled out as a separate class the class II histocompatibility antigen proteins. These were linked with the immunoglobulin heavy chain constant region because a human precursor contained both parts. This large immunoglobulin family was relatively easy to resolve.

The largest and most difficult class to separate contained 653 sequences.

These were viral polyproteins, their components and related proteins. The related proteins included cell division control proteins, hormone receptor proteins, RNA polymerases and proteases. The proteases of the viral genome cleave the polyprotein into its components. There were also repetitive fibrous proteins in this 653 sequence class; actins, keratins, collagens and myosins. This composite family required three threshold shifts to split it into sufficiently small or sufficiently homogeneous classes.

Polyprotein problem

The splitting of the largest high scoring family into manageable subfamilies illustrates a more general problem. This concerned the polyprotein and fusion proteins. Both polyproteins and fusion proteins are composite proteins, each component being a functional protein in its own right. Polyproteins are processed subsequent to synthesis to yield the separate protein components, whereas the components of fusion proteins stay joined. An example of a fusion protein was 'dihydrofolate reductase - thymidilate synthase'. This acted as a link between the dihydrofolate reductase and thymidilate synthase families causing them to be grouped as one. The linking of the families does not reflect a similarity between the dihydrofolate reductase and thymidilate synthase families. In this case the families joined were small and it was easy to see the artificial nature of the join. The polyprotein problem had its most serious effects with the viral polyproteins. Since these contained up to six individual proteins, the linking of families together which they caused was not surprising. This was compounded by the fact that viruses have scavenged the actin sequence from a host. This added a particularly large family into the collection.

Biased protein problem

Spurious links between families can also arise from biased composition of the protein sequences. The cysteine/glycine rich proteins, human metallothionein-IF and wheat agglutinin have a high pairwise similarity score which reflects their unusual composition rather than similarity in pattern. There are many indels in the alignment and little evidence for the cysteines in the different families having similar spacing. Whilst there may be some deeper reason explaining their similar composition, such as the need for a highly disulphide bond cross-linked structure, there is less compelling evidence for relatedness than the scores for such similarities suggest.

Spurious links

The number of spurious links between families caused by these two problems was small enough that manual detection was acceptable. However, because of the method of data reduction, each such link between two families potentially obscures one genuine link between the two families. Thus, were there a weak similarity between some dihydrofolate reductase gene and some thymidilate synthase gene, it would not have been found by this method.

One way round the problem of spurious links potentially obscuring other links would have been to remove the proteins causing the problem. New links might have been found by removing the biased proteins and the polyproteins, and forming the tree afresh using the existing similarity data. Better still would have been subdivision of the polyproteins into their separate components and subdivision of biased proteins into biased and unbiased sections followed by a repeated search with these component pieces. This was not done. There were sufficiently many similarities to follow up as it was.

Known similarities

In many families the names clearly indicated that the proteins grouped together were simply variants of the protein found in different organisms and that the similarity in sequence and function were known.

Sometimes variations in the naming made this less obvious. One less obvious pair was pyruvate oxidase and acetolactate synthase which represent alternative views of the same chemical reaction. Another pair of sequences with strong similarity were N-acetyl neuraminate lyase (EC 4.2.1.52) and dihydrodipicolinate synthetase (EC 4.1.3.3). The EC numbers refer to a hierarchical enzyme classification scheme and indicate substantially different catalytic activities. The synthetase has pyruvate as a substrate, the lyase as a reaction product. Since both must bind pyruvate, the similarity in structure is not particularly surprising. Although the similarity was not mentioned in the 'PSeqip' database annotations, it was later found to have been noted in the PIR database. Both proteins belong to the same protein superfamily. These kind of matches prevent a totally automatic elimination of known similarities based on the protein names alone.

Many similarities between variously named hormone peptides and hormone containing proteins were also found, e.g. one family contained folitropin, luteinizing hormone, pituitary glycoprotein and gonadotropin. Also there were groups of plant toxins and animal toxins which show similarity but which have names that do not immediately indicate that the similarity is known. It is very likely that these similarities are known. They are in any case not particularly surprising. Such similarities were not taken further.

Removing uninformative similarities

Others proteins grouped together that were not of immediate interest represented similarities amongst proteins for which a function was not known. Proteins of unknown function were named variously as unidentified, hypothetical protein and open reading frame. A group of proteins of unidentified function and unusual name were the 'huey', 'duey' and 'louie' mystery proteins from *D. melanogaster*. These lined up against a yeast maltase. This find has been discovered independently and reported in the literature (Heinikoff, 1988). More recent databases, e.g. PIR 26, label these proteins as hypothetical maltases.

Similarities mentioned in the annotations

For each similarity that was not obvious by name and for which the similarity held up after examination of the alignment, the next step was to retrieve the annotation information describing the protein. This stage used the search facility within a text editor "Vecce" on a mainframe computer, emas, which held the annotation data file. For some proteins the unexpected similarity was mentioned in the annotation. For others, the fuller description explained cryptic alternative descriptions for the same protein. These 'finds' could then be eliminated. As the annotations file contained several megabytes of data the searching process proved to be slow and cumbersome.

To help with the task of finding similarities mentioned in the textual annotations the Unix utility 'grep' was used to search for the word 'like' and words starting with 'similar' and 'homolog' in the annotations. This generated a list of proteins whose names alone do not suggest that there is similarity between them, but for which similarity is known. This search was done on the PIR 23 database which was more up-to-date than the 'PSeqip' database.

A faster method for checking in the annotation file entries was still needed. The description of the protein sequences' functions needed to be read, and, where pairs were not obviously related, the references to the literature followed up. The best software available for examining sequence annotation entries was the "Psq" program from the National Biomedical Research Foundation (George *et al.*, 1986) which ran on a VAX computer. Although this program worked only with the PIR databases the 'PSeqip' database could have been converted to this format. Unfortunately space problems on the VAX computer meant that it could not accommodate the database. Transfer of the "Psq" program onto emas would have involved major rewriting to the program. "Psq" makes extensive use of lexical functions which are specific to VAX computers.

Instead, an annotation file cross reference program "Xref" was written which had additional features to facilitate this kind of work. "Xref" had a front end very similar to a text editor. It had the ability to browse through but not to change a file containing results of searches. It recognised within any textual file protein sequence identifiers preceded by a '>'. At any position within the results file, a display of the appropriate sequence annotation entry could be selected. An index file was used to locate the annotations rapidly. The sequence annotation entries, where these were longer than a screenful, could also be browsed through and optionally appended to an output file for later printing.

This program was designed to also be of use in conjunction with files of results produced by searches using "Prosrch". For this use a feature was added that permitted filtering of the display so that only a selected number of lines after each sequence identifier were displayed. The hundred or so "Prosrch" results from a typical search could then be shown at several levels of detail ranging from names alone in order of score, to full display with names, score information and alignments.

For routine use with "Prosrch", "Xref" made checking annotations simpler and more rapid than it would have been with "Psq". The checking could be done on screen from a display of the output file, with a minimum of keystrokes. For use with output from the comprehensive search, a program such as "Xref" was almost essential. Issues of software portability concerning "Xref" are discussed in Chapter 5.

The paper chase

Where the annotation did not reveal that the similarity was known, the next step was to consult literature, taking as a starting point the references cited in the annotations.

This process proved less straightforward than expected. An extreme example of this concerned the bacterial 'host specificity of nodulation' protein 'hsnC'. This protein is one of several essential for the symbiotic interaction of plants and nitrogen fixing bacteria. For a review, see Quispel (1988). HsnC showed similarity to glucose dehydrogenase. The annotations gave references to the papers in which each of these proteins was first presented (Horvath *et al.*, 1986; Jany, 1984). The paper presenting the hsnC sequence postdated the glucose dehydrogenase paper. It seemed that the similarity might not previously have been discovered:

> " The sequences of the hsn gene products were compared to those of other proteins from the GenBank(USA) and from the EMBL data library (Heidelberg). The only homology of any significance was found between hsnA and the acyl carrier protein (ACP) of *E. coli.* " (Horvath *et al.*, 1986)

Further investigation of whether similarity had subsequently been noted involved listing papers citing each of the two papers using science citations indices for all years subsequent to publication. A paper noting the similarity would be likely to cite both of the primary references.

The paper with the hsnC sequence also concerned three other genes from a region of the genome that is of intense research interest. Consequently large numbers of papers cited this paper though many did not refer to the gene hsnC. No paper cited both references. One of the papers (Surin & Downie, 1988) for hsnC also cited another research group. This group determined essentially the same sequence (Debelle & Sharma, 1986). Unlike the research group cited in the database entry, these researchers had noted the similarity of hsnC to ribitol dehydrogenase, an analogous enzyme to glucose dehydrogenase, at the time that they presented their sequence. They had interpreted the similarity in terms of interactions of the bacterium with the plant cell wall.

Two observations were made from this specific example that raise issues a more automated process of literature searching would need to address. Firstly the protein hsnC was entered in the database as 'host specificity of modulation protein', a mistake corrected in later databases. Secondly there was a change of nomenclature subsequent to the discovery of the hsn genes. 'hsnC' was renamed to 'nodG'. This would make a text or keyword based search more problematic. (see also Appendix 1 for discussion of keyword searching).

Concluding remarks

Results scoring greater than 200 generally had alignments giving strong evidence for relatedness. The main exception involved protein whose composition was heavily biased. In such cases scores reflected bias in sequence rather than similarity in sequence pattern. All initially surprising results at this high level of similarity were at some stage found to be known already though similarities to unidentified proteins and between hormones and between some toxins were not followed up. The similarities scoring less than 200 are considered in the next chapter.

The tree reduction method provided an effective way to reduce the volume of data to examine. The tools for browsing the results, particularly the sequence annotations browser "Xref", were essential to analysis of the computer searches' results. This software tool is also useful in examination of results from single database searches. Issues addressed in this chapter may also be of importance in searches of databases with newly determined sequences. One such is the automatic grouping of related sequences. This was crucial in this work. Similar techniques could also be useful in presenting results from single searches.

Chapter 8: Twilight Zone Similarities

Perhaps it is not surprising that the strongest similarities found by the database search were already known. This chapter concerns weaker similarities whose scores are at the boundary between signal and noise, the 'Twilight zone' for sequence comparison (Doolittle, 1990). Similarities discussed in this chapter score between 100 and 200. Except where clearly stated otherwise, the alignments presented in this chapter show similarities that seem not to have been previously noted. None of these new similarities are mentioned in the sequence annotations nor do the family designations as allocated by NBRF, where these sequences are present in the PIR 23 database, indicate a known relationship. Fully establishing that a similarity has not previously been noted is not really possible. Amongst papers concerning the proteins involved in the strong similarities a paper was always found which mentioned the similarity. This was rarely so for the weaker similarities. The similarities thus do seem to be not previously noted ones.

Given the large number of weak similarities, there was felt to be little to be gained by following up the weak similarities which did not seem particularly surprising. For example, there was a weak similarity (score 103) between myo-inositol-binding protein and D-galactose-binding protein. The similarity was weaker than would normally be considered as evidence for relatedness. There were only 12 identities in a stretch of 36 residues. Even if further comparison work could strengthen the confidence in the match, for example by finding additional regions of matching, the known activities of the two proteins are sufficiently similar that merely establishing a connection would add little to biological knowledge.

Similarities in the weaker list, not unexpectedly, include additional examples of ATP binding domains, additional NADH binding domains and more examples of homeobox sequences - families that are clearly present at higher levels of similarity. It is not absolutely clear that every one of these weaker echoes has been previously noted. Examples of sequences containing such patterns are sometimes collected by searching using derived patterns. Using derived patterns captures an 'average' pattern, whereas a new member of a family may be closest to an outlier in the known family - a case which tree reduction catches well.

The weaker similarities had a lower proportion of groups where the similarity was obvious from the name than did the high scoring ones. Similarities that were not surprising and ones which seemed to arise from biased composition in the sequences were crossed off the computer output. This still left a large number of unexplained weak similarities. A disadvantage of a sensitive search is exposed here. It is not clear how much evidence for relatedness weaker similarities provide. This is where it would be helpful to have some method that measures how much evidence for a genuine relationship a particular score gives. In measuring significance it is important to take into account the large number of comparisons made in comparing all pairs of sequences in the database.

Scores to significances

A direct interpretation of the Dayhoff scores as logarithms of odds gives a rough guide to likelihoods of chance matching. A pair of words with Dayhoff score 45 has odds 32000:1 of being signal rather than noise since 10 x *Log* (32000) = 45. The factor of 10 arises because of the scaling of the score table (Chapter 3). This figure relates to a single comparison of two fixed length words.

Odds have to be 10000:1 or better in favour of a match being signal rather than noise for a 'find' made in 10000 comparisons of fixed length words to begin to be significant. This level of odds would be appropriate for regarding a find made in two sequences of length 100 amino acids as significant since roughly 10000 word comparisons would be made.

The number of comparisons made between word pairs when a sequence is compared to a database is approximately the product of the database and sequence lengths. If comparing words from a query sequence of length 490 against a database of size 3,000,000 then 490 x 3,000,000 word comparisons are made. A word match with a score of:

10 x *Log* (490 x 3,000,000) = 92

is then as likely to be from a sequence related at the selected evolutionary distance as it is to be fortuitous. 92 is the 'break even' score for a single database search. For scores above the 'break even' score, each additional ten points in score increases the odds of the match being signal by ten fold. That is, there is a multiplier of ten for odds for each extra ten points in score. Thus the break even score and this 'significance multiplier' are parameters which can be used to estimate significance of a word match with a given score.

Use of the *Log* of the product of sequence and database lengths to adjust for the number of comparisons made has also been suggested by other researchers (Smith *et al.*, 1985). Moreover they provide references which suggest this adjustment is valid in comparisons where word size is variable, which is a closer approximation to the case of comparison by alignment than is comparison using fixed word size.

"Prosrch" uses observed score frequencies to assess significance. In the "Prosrch" program, actual frequencies for some of the higher scoring noise level alignments are collected. These are observed to fall on a negative exponential distribution. Fitting a line to this gives a 'break even' score and a multiplier for the unlikelihood for each additional ten points in score. This gives an empirical model for measuring significance. Unlike the theoretical *Log* odds model, this approach takes into account indels and sequence inhomogeneities.

When a high indel penalty, a penalty of 20 or more, is used with "Prosrch", the parameters which measure significance approach those from the more theoretical model. Since a high indel penalty suppresses indels, this is to be expected. Break even scores and multipliers calculated by "Prosrch" are lower than predicted by the theoretical model, scores being typically 8.8 points lower with an average 8.2 fold increase in significance, rather than tenfold, for each additional ten points.

The lower estimate for 'break even' point can be explained as follows. The 'break even' score calculated by "Prosrch" is the score at which the expected number of random matches is one, rather than the score at which a match is as likely to be noise as to be signal. The more theoretical *Log* odds model thus gives a slightly more conservative assessment of the break even score.

When a lower indel penalty is used with "Prosrch" the significance multiplier decreases. With an indel of 10 the multiplier is around 5.9. The lower factors for lower indels must reflect the increased possibility for random matching arising from increased ease of insertion and deletion.

At low indel penalties the odds method and "Prosrch" give very similar estimates of the break even score. Given this background, it seems reasonable to use the direct interpretation of Dayhoff scores to estimate a break even score for the comparison of every sequence against every other sequence.

For databases of the size of the 'PSeqip' database, a single search with a protein of an average length (300 residues) involves 7.5 x 10^8 comparisons and the break even score is 89. A total database search requires 3.12 x 10^{12} comparisons which corresponds to a break even score of 125.

Break even score for comparison of one sequence against all sequences in the 'PSeqip' database	89
Break even score for comparison of all sequences in the 'PSeqip' database against each other	125

Figure 8.1: Break even scores for different numbers of comparisons. At this score a match is as likely to be signal as noise.

Repetitive sequence problem: Protein-A

Caution is needed in interpreting weaker similarities. It is particularly important when considering barely significant scores to recognise that the score alone tells only part of the story. The problem of biased sequence has already been mentioned. A related problem was exhibited by two proteins which contained repeated subsequences. One protein was the major surface antigen (S-antigen) of malaria, *Plasmodium falciparum*. The other was Protein-A from the bacterium *Staphylococcus aureus*. The score for the match, at 198, was just below the 200 threshold. Such a score would normally imply an alignment showing strong evidence for a similarity. Repeated octopeptides in each protein were aligned by the program. A low score for the match of one octomer pair was multiplied by the repetition in both sequences to give a high score that reflected primarily the fact that both proteins contained repeated domains. That is, the high score related to repetition which could have been present in the proteins for quite different functional reasons.

In the S-antigen, the octomer 'AEARKSDE' was repeated twenty times in succession. Protein-A had the octomer 'EDNNKPGK' repeated eleven times. The similarity score for these two octomers is 7, giving a score of 77 for matching of these repeated octomers. Nine of the S-antigen octomers were thus not aligned with the protein-A octomer repeat. Protein-A also contained a 60 residue sequence repeated four times. The 60-mer contained the 16-mer shown aligning with two of the S-antigen octomers in the alignment below.

```
STASPAP1     - Protein-A (S. aureus)
SSANT$PLAFN  - S-antigen protein precursor (P. falciparum)

               *** * ..*.* *.*.
STASPAP1     : AEAKKLNDAQAPKADN
SSANT$PLAFN  : AEALKSDEAEALKSDE
```

This matching segment has a score of 54. In the alignment found by the comprehensive search, two such 16-mers were aligned against four of the S-antigen octomers contributing 108 to the score.

This example was presented at a talk on sequence analysis to illustrate how comparison algorithms can give artificially inflated scores when repetition is present. In the discussion afterwards, R. Hayward (ICMB Edinburgh) suggested a biological rationale for the similarity. He pointed out that protein-A is part of the bacteria's defence system (Hammond *et al.*, 1984). It binds to the F_c part of immunoglobulins. 'F_c' is the constant domain recognised by phagocytic cells. In *S. aureus*, binding of the F_c component prevents the phagocytes from recognising the bacteria as foreign. In *P. falciparum* the surface antigen could turn out to be a 'molecular mop' that soaks up the immunoglobulins. If the mechanism of binding is the same as in *S. aureus*, then, rather than being the prime target of the immune response, the 'antigen' is sequestering components of the immune response. If the usual interpretation of the protein as a major antigen is incorrect, the mistake is understandable. The radiolabeling antibody tests, which are the data suggesting "major surface antigen", show only that there is a strong association between antibody and the protein.

Further examination of the sequence pair revealed an additional small region of similarity - a pentapeptide 'RNGFI' in the S-antigen and 'RNGFL' in protein-A. There is a 1 in 6 chance of two random sequences of these proteins' lengths sharing such a pentapeptide. This gives some additional evidence for a genuine relationship.

Many variants of the S-antigen are known. These show considerable variation in the repeated sequence. One hypothesis is that repetition in the sequence saturates the immune system to suppress immune responses (referred to in Howard, 1986). A more complex hypothesis than the new 'molecular mop' hypothesis may emerge in time. One factor that casts serious doubt on the new hypothesis is that association of antibody and S-antigen is specific to sera raised against the S-antigen (Howard, 1986). Binding of the F_c component would be expected to be non sera specific. Nevertheless this similarity is intriguing as its score is high and it links proteins both believed to be involved with immune response evasion.

Weak local constraints: Patterned bias

Unlike very strong matching, scores in the range 100-200 are not necessarily suggestive of some similarity in function for the whole sequence. Short shared pattern or longer patterns that are weakly conserved need not reflect a structure that is involved in specific interactions with other compounds. Weak similarity might simply reflect structural aspects of the proteins rather than active sites.

For example, proline is a residue that leads to reduced flexibility of the protein chain since it prevents free rotation about a carbon-nitrogen bond. Glycine, because its small size reduces steric hindrance, gives great flexibility to the chain. One might observe higher incidences of glycine and proline occurring together than chance alone would suggest. Alignments of pairs of glycine and proline rich proteins would then be less significant than their similarity scores suggested since the model for random matching takes no account of the influence of residues on residue frequencies at nearby sites. High scoring alignments of sequences rich in proline and glycine would not necessarily reflect similarities giving clues to function of the proteins. Similarity in sequence pattern in sequences of biased composition could simply reflect local constraints on residues close in the linear sequence that become more evident when bias in composition is present.

Bias and repetition in protein sequence are intimately linked. Any short sequence repeated many times over gives rise to a protein with an atypical compositional bias. Conversely, strong sequence bias greatly increases the likelihood of repeated patterns. Dipeptide repeats are far more frequent in regions where only three residues are used rather than twenty. The mutational properties of DNA are also important where repetition and bias are considered. Once a repeat occurs at the DNA level, it can readily become amplified to a larger numbers of repeats. For this reason, an alignment for sequences with similar repeating patterns may have a score that would normally suggest common origin, when in fact the sequences have arisen independently.

Sequences with biased composition which additionally show repetitive local patterns lead to some of the highest scoring similarities scoring in the range 100-200. DG-rich, QQA-rich and EEKK-rich sequences are three examples where high matching scores between seemingly unrelated proteins are found. It is quite possible to envisage these sequences arising independently of each other.

Viral repetitive proteins

One high scoring twilight zone similarity (score 184) was found between the repetitive glycine and proline rich proteins, collagen (alpha chain, rat), and a protein from a human virus (herpes simplex virus, type I, 34KDA (c) protein). Collagen's repetition gives it its fibrous structure. Repetition in viral coat protein may, as hypothesised for the malaria protein, be part of a virus's defence against the immune system. Mimicking common host proteins would be an additional defence for the virus because host mechanisms which prevent self destruction should also be to the virus's advantage. However, repetition would in itself be a sufficient explanation for the similarity.

Although the muscle protein tropomyosin contains repetitive regions too, repetition is not the explanation for its score, 130, in matching against a protein associated with the viral coat:

```
SXAD9T   - Hexon associated protein (IX) (Tupaia adenovirus)
TMCHS2   - Tropomyosin, smooth muscle (Chicken)

        **   *. **      **** *. ***.*..*..   *  *. * .....  * * *
SXAD9T: TDAATEPSTRQGLNLLRSVTELNESIDELQQKMT-ELEKRLKIMEEKIEEIKLALAN
TMCHS2: TDKLKEAETRAEFAE-RSVTKLEKSIDDLEEKVAHAKEENLN-MHQMLDQTLLELNN
```

The region of tropomyosin in this match shows little sign of repetition. More likely this match reflects a viral scavenging of a host sequence, as was seen with the actin sequence in the previous chapter. In this case subsequent modifications have been more extensive.

Local similarity and local structure

The flexibility of the protein chain at glycine and proline residues was used to illustrate the possibility that local folding constraints, rather than functional similarity, could explain weaker sequence similarities. Can local regions of similar sequence and folding be found in proteins with otherwise very dissimilar sequence and structure? Kabsch and Sander (1984) performed a survey of known 3 dimensional structures to see whether short range interactions between residues necessarily lead to the same local structures in folded proteins. Their purpose was to examine intrinsic limitations in protein structure prediction from local sequence patterns alone. They found examples of identical pentamers in different proteins that did not adopt the same structure. One can be almost certain that these local structures, being radically different, do not have similar function in the folded protein. One possibility would be that the identical pentamers have a role in intermediate stages of the protein folding. With pentamers, chance alone can explain the occurrence of identical sequences in functionally distinct proteins. No functional explanation for the similarity need be found.

Two identical decamers in distinct proteins are unlikely to occur by chance. If two identical decamers did not adopt similar structure in two different folded proteins some explanation would be required for the identical sequence occurring. One possibility would be that the segments adopt similar structure at an intermediate stage of folding.

Amongst the many similarities found, one find which is just significant for a single search (score 103) but not for an entire comparison occurs between a bacterial phosphofructokinase and a simian myoglobin:

```
KIBSFF - 6-phosphofructokinase (Bacillus stearothermophilus)
MYMQN  - Myoglobin (Night monkey)

        *.*. *.*. *.*****.  *  .**
KIBSFF: KTEEGEKKGIEQLKKHGIQGLVVIGG
MYMQN:  KSED-EMKASEELKKHGVTVLTALGG
```

Unusually, since fewer than 2% of proteins have had their structure determined, both sequences are of known structure (for the myoglobin a very close sequence analogue exists). Like Kabsch and Sander's examples this similarity contains an exact matching pentamer pair but it also has additional flanking similarity. In myoglobin the region lies between the D4 and E16 helices. Examination of the crystal structure using the program "O" (Jones *et al.*, 1990) on an Evans and Sutherland EV/PSX shows that the similar regions in sequence are different in structure. Both involve some helix and some random coil but the selected segments of the structures do not superimpose. This region is a possible candidate for a similarity reflecting structural intermediates in folding. The sequence similarity shown here is weak and though stronger than the pentamer matches, could be a chance match. The structural comparison does, however, show that low scoring similarity is not enough to establish that proteins or even segments have similar structure. With low alignment scores one requires additional evidence for sequence relatedness.

Multiple matching: An AMP binding pattern?

The alignment score is not the only factor in assessing significance. Repetition and bias tend to reduce confidence that an alignment is as significant as its score suggests. Other factors can increase confidence.

If segments from two proteins match with a third protein by chance these matches are most likely to occur in different regions of the third protein. One factor that might increase confidence in an alignment is finding the same region being matched by several different proteins. The ATP binding motif gives rise to matches of this kind. Any other short motif pattern that is found in otherwise dissimilar proteins would also give rise to multiple matches to one region of a protein. In the following example two proteins match a third in the same region. Both the pairwise similarity scores for matching to adenylate cyclase are below the break even score of 125. For the tail assembly protein the score is 101, for trans zeatin the score is 107.

```
SVATAI$LAMBDA - Tail assembly protein i. (Bacteriophage lambda.)
SCYAA$ECOLI   - Adenylate cyclase. (E. coli)
TIPTZSP1      - Trans zeatin. (Agrobacterium tumefaciens)
```

The best region of matching in a more extensive match is shown here:

```
                *+ ++**+*+ +* ++
SVATAI$LAMBDA: YGDLQRFGRRIDLRVK
SCYAA$ECOLI  : YRNLIRFARRNNLSVS
TIPTZSP1     : YRCAIRFARKHDLAIS
```

The line above the alignment emphasises the additional evidence for relatedness. Locations where all three sequences agree are shown by '*', locations where two out of three agree by '+'.

Finding local similarities of borderline significance that overlap is not all that surprising under the hypothesis of unrelated proteins. The database search produced 720 linking families of, on average, 3 proteins each. There were many opportunities for such an overlap to occur by chance. Finding overlapping low scoring matches is not in itself of great significance.

The situation is different if such multiple matching is accompanied by knowledge that the proteins interact with similar molecules. In that case the hypothesis that the region is involved with the specific interactions is of biological interest. Although we measure significance numerically, we are actually more interested in similarities that lead to new biological understanding.

Adenylate cyclase catalyses the conversion of ATP to cyclic adenosine monophosphate (cAMP) (Aiba *et al.*, 1984). Trans zeatin catalyses the addition of isoprenols to adenosine monophosphate (AMP) (Akyoshi *et al.*, 1985). The tail assembly protein i (Sanger *et al.*, 1982) takes part in the initiator complex for lambda tail formation. A possible role for AMP in this initiation process does not appear to have been investigated.

Cyclic AMP has diverse roles. It is a second messenger for hormone action, is involved in glucagon metabolism and can stimulate active transport of ions. Thus, like ATP, AMP is a candidate for having a binding motif in a range of different proteins. The similarity in combination with the biochemical information lead to the hypothesis that the region shown binds AMP.

How should one proceed in this case? The known biochemistry suggests that the similarity is of interest, but the similarity scores are too weak to do more than suggest the hypothesis. If the weak pattern binds AMP, corroborating this by further sequence analysis and characterising the pattern better is likely to be considerably harder than characterising the strong ATP binding pattern.

A first and simple step would be to use each of the three protein sequences in turn to search the protein database and collect weaker matches to this region. These weaker matches might identify proteins with known interactions with AMP. To make further progress, a survey of current literature on cAMP and on the cytokinins, the AMP derivatives partially synthesised by trans zeatin, would be important. Also literature on the biochemistry of the sequences involved in weaker matches would need to be examined. This combined background would give information on the likelihood of the weaker matches having previously unsuspected interactions with AMP. For example, weak similarity to adenylate cyclase in the given region is shown by the toxin ricin, score 82, and by agglutinin, a plant lectin, score 90 (S.J. McQuay, ICMB Edinburgh, unpublished results). If for biochemical reasons interactions with AMP could be important for these proteins too then the similarities would tend to confirm the AMP binding hypothesis. This possible approach shows the importance of biochemical knowledge in sequence analysis work.

A tantalising additional piece of information that could be important in such a study comes from a review of cAMP dependent protein kinases (Taylor *et al.*, 1990). In these protein kinases a regulatory subunit inhibits the enzyme's phosphorylase activity by occupying the active site, thus preventing access by other substrates. Binding of cAMP to the kinase leads to dissociation and activation in a manner yet to be elucidated in detail. Of particular interest then is observation of the sequence 'RFDRR' in the R-II alpha regulatory subunit of bovine cAMP dependent protein kinase for this pentapeptide occurs at the specific site in the regulatory subunit that inhibits kinase activity and is identical in four of the five residues to that of the lambda protein in the putative AMP binding pattern.

Conserved cysteines: Two plasma proteins

In the example in this section, examination of the alignment increases confidence in the match. Here seven cysteines in a segment of thirty-two residues are conserved. Because cysteine can form disulphide bonds, structure could largely be conserved even though residues in the longest sections between the cysteines differ. This similarity has a score of 109, but the kind of matching increases confidence in its validity.

```
MUSPC1BP1     - Plasma-cell membrane protein. (Mouse)
P1SGHU2V      - Vitronectin precursor, serum spreading factor. (Human)

                ****** *    .  * **  *     ** *.
MUSCPC1BP1    : SCKGRCFERTFSN..CRCDAACVSLGNCCLDF
P1SGHU2V      : SCKGRCTEGFNVDKKCQCDELCSYYQSCCTDY
```

The fact that both proteins are also plasma proteins increases confidence too.

Other cysteine rich domains in proteins are known. 'Kringle domains' are cysteine rich triple-looped disulphide cross-linked domain found in serine proteases and plasma proteins. Epidermal growth factor (EGF) domains are also cross linked and cysteine-rich. In examples of these domains, the pattern of cysteine residues is conserved.

The region of vitronectin shown here has been identified as a 'somatomedin-B' domain (Jenne & Stanley, 1987). Somatomedin-B is a growth hormone dependent serum factor (Fryklund & Sievertson, 1978). For the plasma cell protein the region's similarity to somatomedin-B does not appear to have been identified. Instead to relate the protein to other proteins, Van-Driel and Golding (1987) drew analogies between the plasma-cell protein, which is selectively expressed on the surface of antibody secreting cells, EGF receptor and transferin receptor, also membrane proteins. These analogies were based on cysteine content and on hypothesised orientation and position at the cell membrane surface. Van-Driel and Golding singled the thirty residue sequence from the plasma cell protein out as being of special interest for it is cysteine rich, occurs as an imperfect tandem repeat and is thought to take up a position immediately external to the cell membrane.

The vitronectin sequence similarity showed regions of good matching separated by a region of poor matching, though the conservation of cysteine residues was a more striking feature. Even where cysteine residues are not involved, clumping of good matches into short regions increases confidence in a low scoring alignment. Since proteins are folded structures, similarities in structure may not be reflected by similar sequences in unbroken contiguous stretches. The Type III algorithm is designed only to detect unbroken contiguous local similarity. Type III alignment only finds broken similarity where regions flanking a region of poor matching each more than compensate for the poor score of the intervening region. Scores returned by the algorithm reflect the similarity over the whole region matched. Two good regions each scoring 90, separated by a region scoring -70 would lead to a combined score of 110. If a region scoring -100 separated the two good regions, the protein similarity would score just 90, one or other of the matching regions being presented in a local alignment. In both cases the score to the biologist is closer to 170. Automatic methods to link islands of good matching together which incur only a small penalty for intervening regions of poor matching are needed. The automatic linking of islands of good matching was seen to be important in alignments in Chapter 6. It also seems to be important in scoring weaker matches. An example where clumping of similarity in two islands increases confidence in the alignment will be given shortly.

Similarities across taxonomic boundaries.

Of particular interest in this study were similarities between very different organisms. Similarities between two proteins in different mammals are more likely to be known to researchers for other reasons than similarities between proteins in insects and mammals or similarities between proteins in animals and plants.

Similarities between plant and animal are particularly likely to be of a fundamental nature, given the long time since they shared a common ancestor. The fundamental molecular machinery for nucleic acid and protein polymerisation are similar in plants and animals. It is no surprise then to find strong sequence similarities between ribosomal proteins of plants and animals. Other sequence similarities that cross taxonomic boundaries may also reflect fundamental biochemical processes.

Two pathogenesis related proteins

A similarity which links plants and animals is shown below. Though this similarity scores only 103, it is present in two regions each with a high density of matching and this increases confidence in its validity.

```
DNMS53   - Cellular tumour antigen p53. (Mouse)
VCT014   - Pathogenesis-related leaf protein p14. (Tomato)

         .******  *.           ** .* * ***  * .  **..*
DNMS53: PVQLWVSATPPAGSRVRAMAIHKKSQHMTGVVRR-CPHHERCSDG
VCT014: AVQLWVSERPSYNYATNQCVGGKKCRHYTQVVRLGCGR-ARCNNG
```

Moreover, the two proteins' functions suggest that this sequence similarity may be the basis of a functional similarity. The leaf pathogenesis related (PR) protein is expressed at elevated level in a pathophysiological non pathogen specific response (Lucas *et al.* 1985). The mouse tumour protein is expressed at elevated levels in cells transformed by a variety of mutagens, X-rays, chemicals and viruses (Zhakut-Houri *et al.* 1983). The mouse tumour protein may have a role in preventing cancerous transformation since mutant forms of the protein predispose cells to transformation and elevated levels of functional forms can act to suppress it (Finlay *et al.*, 1989).

The expression of specific proteins at elevated levels when cells are subjected to abnormal physiological stress has a precedent in the heat shock proteins. These were first observed expressed in response to increased temperature, but other stressors can also induce them (Lindquist & Craig, 1988). Remarkable conservation of sequence in heat shock proteins has been found across the plant and animal kingdom, the best example found by the comprehensive protein comparisons being between *D. melanogaster* and soy bean, *Glycine max*.

Some heat shock proteins are 'sigma factors'. Sigma factors regulate the expression of genes. They are interchangeable components of RNA polymerase, the protein complex responsible for making copies of portions of DNA to be translated into protein. The sigma factors determine at which specific DNA patterns the protein complex which transcribes DNA initiates its replication. Different sigma factors have different DNA recognition patterns. At elevated temperatures there is a change in expression of sigma factors and a global shift in the expression of genes. The leaf pathogenesis protein, however, shows no similarity to known sigma factors and is known not to be expressed in response to heat shock. The proteins might, however, have important interactions with the conserved heat shock system, for the p53 antigen has been found to bind to the heat shock protein hsc70 (Young & Elliot 1989).

Shortly before this thesis was completed, a repeat search was made using "Prosrch" with the tomato PR protein on a more up-to-date database, PIR 26. This uncovered additional evidence that a protein of importance to plants and animals is involved.

```
B31085: Antigen 5-3 precursor fragment. (Bald-faced hornet)

        .***  .  .* *   .. *** * ** . *  *
B31085: IGCGSVKYIENNWHTHYLVCNYGPAGNYMDQPIY
VCT014: LGCGRARC-NNGWW--FISCNYDPVGNWIGQRPY
```

The match shown here (score 96) is to the right of the match with the cellular tumour antigen and overlaps by ten residues.

The similarity of PR proteins to the cellular antigen appears not to have been noted (Kauffmann *et al.*, 1990). The similarity to the venom antigen has been noted, but the biological significance was, and still is, unclear (Fang *et al.*, 1988; King *et al.*, 1990). Possibly the similarity to the mammalian tumour antigen may shed more light on this surprising similarity.

Protein formation and folding

A polymerase, in this case the ribosomal polymerase, figures in an interesting similarity between organisms separated by a wide taxonomic divide. This example is a similarity between prokaryotes and eukaryotes. The alignment has score 122. A multiple sequence alignment for this similarity was given at the start of Chapter 6.

```
ISRTSS   - Protein disulphide isomerase. (Rat)
R3EC2    - Ribosomal protein S2. (E. coli)

        ***.  * **.  * *.*. ..  *.. .* * . ******.
ISRTSS: IFGGEIKTHILLFLPKSVSDYDGKLSNFKKAAEGFKGKILFI
R3EC2 : IFGARNKVHIIN-LEKTVPMFNEALAELNKIA-SRKGKILFV
```

Protein disulphide isomerase is believed to be responsible for ensuring that proteins in the lumen of the endoplasmic reticulum fold correctly and form the correct set of disulphide bonds (Freedman, 1989). This similarity suggests the hypothesis that the ribosomal protein has some previously unsuspected function in protein folding.

Arginosuccinate and two viral proteins

Two viral protein matches are intriguing in that they independently show similarity to proteins involved with arginosuccinate. Each similarity in itself is strong enough to be evidence for a genuine relationship. The first has score 147:

```
SASSY$HUMAN - Arginosuccinate synthase. (Human)
SCOA2$JCPOV - Coat protein VP2 JC. (Polyomavirus)

             **  * * *    ..*. ..   *.*.  . ..*  * ..** ****
SASSY$HUMAN: WVDIEEITRNTVREIGYVHSDMGFDANSCAVLSAIGKQSPDINQGVD
SCOA2$JCPOV: WVS-EAI-RTRPAQVGFCQPHNDFEASRAGPFAA-PKVPADITQGVD
```

The second a score of 130. Only the best parts of this second alignment are shown here.

```
P1CYCHD     - Delta crystallin [Arginosuccinate lyase family]. (Chicken)
REO3S1CP1   - Sigma 1 protein prepetide. (Reo virus)

             **  .* *** *.                            **. *   . . ** ** *
P1CYCHD    : LEKILSGLEKISE ..(120 residues omitted).. RITVLPLGSGALAGNPLEI
REO3S1CP1  : LESRVSALEKTSQ ..(120 residues omitted).. RISTLERTAVTSAGAPLSI
```

Phospoenolpyruvate regulated sugar transport

The final similarity in this chapter is between a bacterial phosphocarrier protein and a protein called patatin. It has a score of 125.

```
WPSAHP   - Phosphocarrier protein HPR (S. aureus)
POTPHO   - Patatin (Potato)

         **.* *  .** **    *  * * *** * . *    *.*.
WPSAHP:  ATMLVQTASKFDSIDQGGYDSMQLKSLGVGKDEEI-TIYSAD
POTPHO:  ATKLAQVDPKFASIKSLNYKQMLLLSLGTGTNSEFDKTYTAE
```

Patatin is the dominant protein of potato tubers. It has a lipid-acyl hydrolase activity. It is differentially expressed in different parts of the potato plant, expression being sucrose regulated and greatest in the potato tuber (Wenzler *et al.*, 1989; Jefferson *et al.*, 1990). The bacterial phosphocarrier protein is part of the phosphoenolpyruvate sugar phosphotransferase (PTS) system involved in regulation of carbohydrate uptake (Reizer *et al.* 1988). The PTS system is basic to a wide class of bacterial cells (Saier *et al.* 1985). Elements of the system could predate the divergence of plant and bacterial lines. Horizontal transfer of genetic information, that is transfer of genes via for example plasmid DNA in bacteria, is also a possibility that cannot be ruled out. In this context, since transposons can mediate genetic rearrangements, it is intriguing to note that the only transposon like sequence so far discovered in potato is immediately prior to an inactivated patatin gene (Kostertopfer *et al.*, 1990).

Concluding remarks

This chapter shows factors which can increase and decrease confidence in the significance of an alignment. Similarity scores are only one factor in the assessment of significance. Ultimately biological interpretation is crucial. Even the strongest of similarity is merely a curiosity if no biological interpretation can be found, whilst a weak similarity is of value if it leads to a hypothesis that can be experimentally validated. Biological interpretation plays the most important role in assessment of whether sequence similarities are worth pursuing further. Computer analysis on its own is not enough.

Each of the alignments presented in this chapter is of potential interest to biologists. It is intended that the similarities involving the bacterial phosphocarrier protein, the tomato pathogenesis related leaf protein and the mouse plasma cell membrane protein will be communicated to researchers who work on these proteins. If these researchers find this information helpful and of interest then this will increase interest in the other similarities presented which are likely to be harder to evaluate.

Chapter 9: NWS Variants

Coding regions and frameshifts

Protein database searching programs are frequently used by researchers who have recently determined a DNA sequence. This DNA could code for a protein. They wish to find whether the protein potentially coded for by the DNA is homologous to a known protein.

Before the computer search can begin, the DNA sequence must be translated into protein sequence. There are three frames in which a single strand of DNA can be translated. These correspond to different starting positions for the grouping of adjacent triplets of nucleotides into codons. If the complementary strand is taken into account there are six possible frames. Usually only one frame codes for a protein in a protein coding region. Translation of codons in this frame gives a protein sequence to test against the database. A translation of all six frames can help in determining which frame is most likely to be the coding frame. An example is shown below. This example is only a fragment of a six frame translation that covers all 674 nucleotides of a DNA sequence from *D. melanogaster*.

```
(Linear) MAP of: Mal.Dna  check: 6903  from: 1  to: 674

                          November 12, 1990  09:19

           ttgcattggagccagcgctgtaagtatggctccctgctggcgggcacaatcggaggcacc
        30 +---------+---------+---------+---------+---------+--------- 89
           aacgtaacctcggtcgcgacattcataccgagggacgaccgcccgtgttagcctccgtgg

a           C  I  G  A  S  A  V  S  M  A  P  C  W  R  A  Q  S  E  A  P
b            A  L  E  P  A  L  *  V  W  L  P  A  G  G  H  N  R  R  H  R
c          L  H  W  S  Q  R  C  K  Y  G  S  L  L  A  G  T  I  G  G  T
        30 +---------+---------+---------+---------+---------+--------- 89
d           A  N  S  G  A  S  Y  T  H  S  G  A  P  P  C  L  R  L  C  R
e            C  Q  L  W  R  Q  L  Y  P  E  R  S  A  P  V  I  P  P  V  P
f          Q  M  P  A  L  A  T  L  I  A  G  Q  Q  R  A  C  D  S  A  G
```

Figure 9.1: Six frame translation of a DNA sequence made using the GCG "MAP" program (Devereux et al. 1984).

In this six frame translation, the DNA sequence and its complement are shown first. Translations of forward frames, a to c, and complementary frame translations, d to f, which should be read from right to left, are shown below.

Of the sixty four possible triplets of bases, three triplets do not code for an amino acid. These three 'stop codons' indicate to the ribosome the end of a protein coding region. Where a codon codes for a 'stop codon', a star is shown in the translation. Likely protein coding regions are the longer stretches free from these stars. These are called open reading frames.

Confidence that a particular open reading frame codes for a protein is increased if the frame uses certain codons in preference to others. Transfer RNAs (tRNAs) are the molecules which carry amino acids to the ribosome and which directly interact with the codons in RNA that is being translated. In every organism some tRNAs for a particular amino acid are more abundant than others. The corresponding codons are used preferentially. This 'codon preference' is the basis for some methods for detecting likely coding regions (Gribskov *et al.* 1984). The methods rely on knowing which codons are preferred in the organism, since codon preferences differ in different organisms. The method works well for highly expressed proteins for which the pressure to use the preferred codons is high. For proteins expressed at a lower level, codon preference is a less reliable indicator. In the absence of a clear choice of open reading frame, the researcher may wish to search a protein database with three or possibly six translations of the DNA.

Problems arising from frameshift errors

Problems in searching arise when there are frameshift errors in the DNA data. An extra or missing base will lead to triplets downstream of the error being out of frame, incorrectly translated and an abrupt end to any detected region of similarity between the translation and a sequence in the database. More importantly, such frameshift errors are also a cause for failure to identify real open reading frames. The shift can make a stop codon from another frame appear to terminate a coding region, so making the coding region appear shorter and hence less significant than it really is. Frameshift errors also interfere with detection of codon preference. The methods to find codon preference consider codon usage averaged over a number of codons. With a frameshift, the codons considered will include codons which are not in fact part of the coding region.

"Fradho"

Molecular biologists frequently require database searches with protein sequences deduced from DNA data. Protein purification and sequencing is a far more complex and labour intensive process than is DNA sequencing. In fact, the overwhelming majority of protein sequences now being determined are sequences deduced from DNA. A method of protein database searching that could accommodate frameshift errors in the DNA is highly desirable. It could extend a matching region that has ended abruptly due to a frameshift error in the DNA data. The extended match would have a higher score that took into account the additional similarity. By improving the ability to detect signal, the sensitivity of the searching should be improved.

A second reason for accommodating frameshifts is that ribosomal DNA translation can occasionally involve frameshifts (Atkins *et al.*, 1990). Frameshifting in translation may be more widespread than currently thought, given that algorithms for sequence comparison are not designed to detect such shifts. The presence of sequencing errors in the data is the more compelling of the two arguments for comparison that can accommodate frameshifts.

A program, "Fradho", was written to search protein databases using a DNA query sequence and allowing for frameshifts. The program extends the normal Type III algorithm by treating information in a hierarchical fashion. DNA bases are the lowest level of the hierarchy. These are grouped in triplets to form the level above in the hierarchy. The program scores similarity at the protein level of the hierarchy whilst not losing the ability to deal with errors at a lower level in the hierarchy. "Fradho" uses multiple path matrices to do this.

The "Fradho" program performs three Type III comparisons simultaneously. Each comparison is for a different reading frame of a nucleic acid sequence. These three protein sequence comparisons use the sensitive Dayhoff amino acid scoring scheme. The three comparisons are interdependent. A missing or extra base corresponds to switching frame and a score in a cell of one path matrix influencing the score in a cell of another. Each such switch between frames incurs a penalty, much as a gap in an alignment incurs a penalty. The decision to postulate a change of frames is objective in the same sense that postulation of insertions or deletions in a normal Type III alignment is objective. Switching between frames occurs in such a way as to maximise the overall level of matching as measured by the score.

"Fradho" can operate in single sequence pair comparison mode or can compare a DNA sequence against an entire protein database. A match found by "Fradho" could be shown using a three frame translation of the DNA by placing dashes after each amino acid involved in matching as in figure 9.2. Frameshifts then show up clearly where the dashes move from one line to another. Potential sequencing errors can then easily be identified.

```
        30 +---------+---------+---------+---------+---------+--------- 89
           ttgcattggagccagcgctgtaagtatggctccctgctggcgggcacaatcggaggcacc
                                    ??                      ?
a           C  I  G--A--S--A--V--S--M  A  P  C  W  R  A  Q  S--E--A--P--
b             A  L  E  P  A  L  *  V  W  L  P  A  G  G  H  N  R  R  H  R
c          L  H  W  S  Q  R  C  K  Y  G--S--L--L--A--G--T--I  G  G  T
        30 +---------+---------+---------+---------+---------+--------- 89
```

Figure 9.2: Three frame translation showing possible locations of frameshifts.

A more condensed form of output, as used by "Fradho", is shown below. This shows the sequence aligned to, as well as the predicted sites of frameshifts:

```
:>P1;DEECIP
:IMP dehydrogenase (EC 1.1.1.205) - Escherichia coli
Score 127 at 38 173

    ***** - **.****+ *.***  . .*   * ******* ** .* . *
 38 GASAVSatGSLLAGTaSEAPGEYFFSDGVRLKKYRGMGSLEAMERGDAKR 182
173 GASAVM-VGSMLAGT-EESPGEIELYQGRSYKSYRGMGSLGAMSKGSSDR 221
```

Figure 9.3: Mixed alignment containing DNA and protein sequence as produced by "Fradho". The upper sequence is the query sequence, mostly translated, but with occasional bases shown in lower case at frame shift positions. The lower sequence is the IMP dehydrogenase sequence found in the database. Numbers for the upper and lower sequence refer to base pair and amino acid positions respectively.

This mixed alignment shows amino acid residues in capitals and bases in lower case. Wherever there is a frameshift the alignment switches temporarily into DNA mode. The '-' and '+' above the line indicate missing or extra bases. '*'s and '.'s indicate identity and conservative substitution as in normal protein sequence alignments. Since a search of a database may produce many alignments, it is convenient to present only the condensed form. To relate this mixed alignment to the complete DNA sequence requires a three frame translation. "Fradho" produces one at the start of each list of results.

Test with new sequence

The alignment shown in figure 9.3 was from the first test of "Fradho" using a newly determined sequence. I am grateful to R. Slee (ICMB Edinburgh, personal communication) for this sequence data at an early stage of sequencing and prior to publication. The alignment predicted an omitted base and an additional base in the DNA sequence. Both of these were sequencing errors located in a part of the gel which was particularly difficult to read. By compensating for these the program was able to demonstrate a convincing relationship between the DNA query sequence and the protein IMP dehydrogenase from the PIR 23 database.

Test with DNA sequence from databases

Individual DNA sequences from DNA databases were also compared against the protein database using the new algorithm. Examples were chosen which were thought to be likely candidates for frameshifts, sequences from transposable elements, a sequence which is translated in two overlapping reading frames and a locally repetitive sequence, a mating hormone from yeast, were tested. None of these searches found strong matches to proteins other than those that were already known to be homologous. "Fradho's" alignments suggested frameshifts in regions of poor matching. As measured by the score, these alternative alignments were only marginally preferable to those for unframeshifted sequence. With a higher penalty for frameshifting no frameshifting was predicted in these regions. However, for the purpose of this test it was correct to use a low frameshift penalty so that even weak evidence for a frameshift would be shown.

Test with longer sequence of known homology

To further the development through criticism of the program, a beta test version was sent to D. Rouch (School of Biological Sciences, Birmingham University, U.K.), a molecular biologist with strong interest in computational methods of sequence analysis. He demonstrated the value of the program at an early stage in sequencing where a homologous protein is already known. This use was not initially considered by this author. At the time the program was seen primarily as a new method for sensitive database searching. A particular test case used by Rouch was a six kilobase DNA sequence, Tn917, coding for a protein essential to the integration of transposable elements. This sequence, determined in the laboratory of D.B. Clewell (Michigan University, U.S.A.) was tested for frameshifts against the known homolog Tn413. "Fradho" found five potential frameshift errors. Four of these were seen to be sequencing errors when the gels were reexamined. Each of the actual errors was within four base pairs of the site of the error predicted by "Fradho". The fifth error predicted by "Fradho" indicated that the C terminal region of the protein extended beyond the point where it had previously been thought to end. Further sequencing confirmed this result and lead to publication of a sequence correction (An & Clewell, 1991).

There is of course a danger in using this comparison method for data correction. The normal process of DNA sequence checking involves re-sequencing the DNA and its complement several times. This results in a reduction of all types of sequencing errors, both in regions of homology and outside such regions. If a criterion of consistency with already collected data were used as a substitute, the quality of sequence data being collected would be decreased. Sequence would only be reliable where homology existed to independently sequenced data. The tool therefore has serious limitations as a method for sequence correction.

Test with simulated shifts

As well as the practical tests of "Fradho", experiments have been performed with simulated erroneous sequences to compare the performance of "Fradho" to protein-protein comparison programs. In these experiments the test of the GCG program "Wordsearch" was performed by Rouch using the Daresbury SEQNET facility. His tests compared "Fradho" to a wider range of programs than here (manuscript in preparation). The test method used a human haemoglobin sequence which had a varying number of artificial frameshifts introduced into it. Haemoglobin is a particularly suitable example for this kind of test as there are over 400 homologues exhibiting a gradation in similarity from close to more distant relationship.

The performance could be measured by counting globin sequences recovered before the first reported non-globin sequence. However, this measure is sensitive to the score of the highest scoring outlier from the noise distribution. Also we are interested in all globin similarities that have been separated from noise similarities sufficiently to be reported. A measure which better reflects detection of similarity in practice is the number of sequences recovered before noise starts to dominate signal. The statistic for this located the position in the sorted list of results at which half the sequences were non-globins. The number of globin sequences recovered before this halfway point was the measure of performance used.

Determination of the 'half way' point used the fraction of globins in groups of ten consecutive sequences. In practice groups larger than ten gave virtually identical results. When using the protein-protein comparison programs the best result for the three searches using the three different frames was recorded. In all cases the protein database used was PIR 23.

The graph in figure 9.4 plots sequence recovery against number of frameshifts. The three lines shown are for "Fradho", "Prowl" - the implementation of the Type III algorithm without frameshifts, and "Wordsearch" the GCG package's word based method. They show that with no frameshifts "Prowl" does fractionally better than "Fradho". Noise levels are higher in "Fradho". This is a result of the increase in score of noise alignments through (a) allowing frameshifts and (b) increased numbers of comparisons from comparing three frames rather than one frame. This is more than compensated for by improved signal recovery for frameshifted sequence. The subsequent level response of Fradho with increasing number of frameshifts shows that recovery of sequences is virtually unaffected by frameshifts. Frameshifts only significantly affect recovery at a very high density with frameshifts occurring every twenty bases. This behaviour is clearly superior to "Prowl". "Prowl" shows a continued decrease in sequences recovered, a result of progressive shortening of regions of homology.

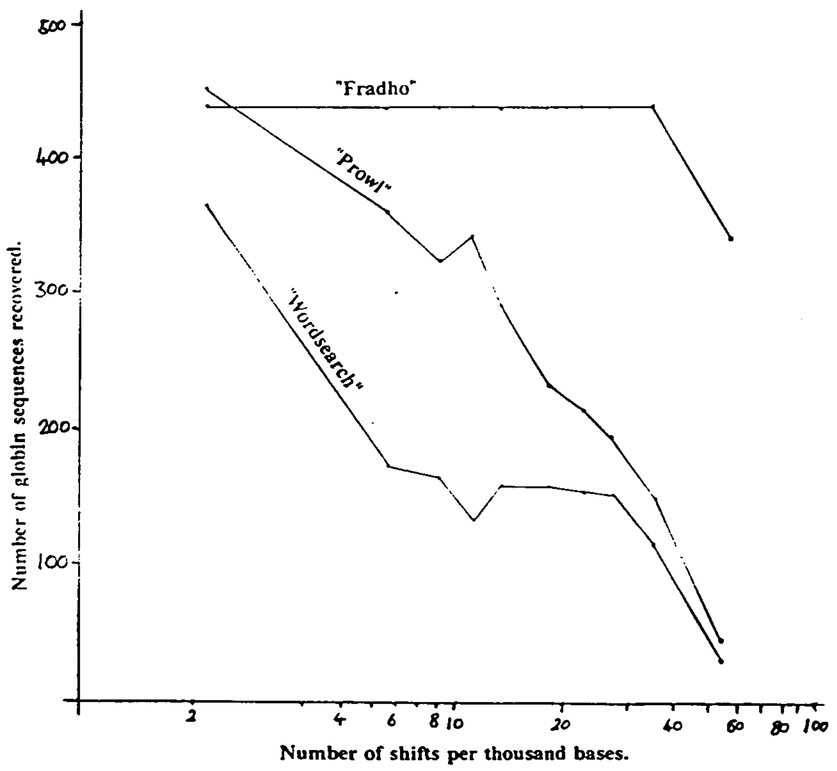


Figure 9.4: Graph showing globin sequence recovery for varying numbers of frameshifts with three different searching programs.

"Wordsearch" shows markedly lower recovery than either of the two Type III algorithms. It has a section of nearly level response but with relatively few sequences being found. Here it seems that the stringent requirement for exact matching words has eliminated all but the most strongly related sequences. Since these have many words in common with the query sequence, it takes a considerable level of disruption to remove these matches too. A more precise explanation of the level behaviour of this program looks in more detail at how

"Wordsearch" works. "Wordsearch" is able to accumulate scores from several regions. With regularly spaced frameshifts, protein translations in just one frame show regular islands of homology. As the number of frameshifts increase, the islands become smaller but the number of islands increases. The average level of sequence similarity measured over the whole sequence stays roughly the same. The results once again show an important weakness in the local homology algorithm. The fact that its scoring is based on a single best region means that it does not score for additional similarity from widely separated regions. The local algorithm is unnecessarily restrictive in basing its score on the result for the single best island alone. A suggested method to overcome this weakness of the local algorithm is discussed later in this chapter. Although the "Fradho" program is also a local algorithm, for the haemoglobin example, the local regions of similarity it finds happen to extend over the whole sequence. For this reason "Fradho" does not suffer from this 'isolated islands' effect in this example.

Types of frameshift error

So far no details of the manner in which the "Fradho" program scores insertions and deletions have been given. The program distinguishes four kinds of frameshift. A frameshift occurs when one or two bases from the DNA line up against an amino acid or against a gap in the amino acid sequence. The table below shows the four possible types of frameshifting step:

DNA	Protein	Description in terms of error in DNA
ag	K	Missing base
a	K	Two missing bases
a	-	Extra base
ag	-	Two extra bases

Figure 9.5: Table showing examples of four possible frameshift steps

The simplest scoring scheme has the same penalty for each of these. However, on the assumption that errors are much more likely in the DNA sequence than in the protein, a progressive penalty with the penalties for two missing or two added bases greater than for one missing or one added base in the DNA is more appropriate. The progressive scoring scheme was used for the examples presented here.

One might also wish to consider scoring schemes which treated errors in protein and DNA on an equal footing, that is a two base omission scoring the same as a one base insertion and a two base insertion scoring the same as a one base omission. The algorithm is 'robust' with respect to the frameshift parameters. That is, changes to the parameters have only slight effects on the recovery of related sequences. For a typical alignment the number of predicted frameshifts is small, four or five frameshifts per hundred residues. Changes to frameshift penalties have a very small percentage effect on the score. The choice of scoring matrix has a far greater influence on the overall score than does the choice of frameshift penalty. The essential new property of the algorithm is that it can accommodate the DNA errors at all. By doing this it can combine matching regions that would otherwise not be seen as originating from the same open reading frame, so improving the ability to detect signal.

The scoring for base insertions and omissions takes no account of the kind of bases inserted or omitted. Two bases aligning against an amino acid always incur the same penalty irrespective of the bases and amino acid involved. 'aa' scores the same against 'V' as it does against 'K' even though none of the four codons for 'V' involves two 'a's, whereas both codons for 'K' do. In view of the comments on alternative frameshift scoring, very little gain can be expected from taking account of such differences.

Use of DNA database

Would it be better to search DNA sequence against a DNA database to detect frameshift errors? Reasons for preferring a comparison at the amino acid level concern discriminating power. As was shown in Chapter 3, scoring protein similarity in protein coding regions gives better discrimination of signal from noise than scoring the DNA. Moreover, the DNA database contains a large proportion of DNA that does not code for protein. By restricting the search to sequences known to encode protein, chance matching from non coding sequence is eliminated. Naturally for non-coding similarities a search using DNA against the DNA database is required.

Practical Issues: Portability

Prior to "Fradho" all programs developed on the PC had been written in Turbo Pascal. A criticism was made that this restricted use of the programs to the PC. Turbo Pascal has various extensions to Pascal which are not supported in versions of Pascal running on other machines. The most important of these is the ability to define 'units'; modules of code which fulfil specific classes of functions. For example, most of the programs written for this thesis share a module for reading sequence data which automatically works out which of four standard formats is being used.

Whilst it would have been simpler to write "Fradho" using previously written Turbo Pascal units, the decision was taken to write "Fradho" in C. Early incarnations of the code ran on both PC and on a network of Sun workstations. However, the superior development environment of the PC lead to most of the development and debugging being done on the PC. Development of both versions in tandem involved regularly transferring programs between machines and writing conditionally compiled code to cope with different operating systems and memory constraints. This slowed development and testing. Little use was made of the Sun workstation version and it was decided to develop only the PC version further.

Practical Issues: Long running times

A major problem with "Fradho" was the long running times for searching. Sequences of a thousand bases required that the program run for twelve hours. This was a consequence of the greater complexity of the cell operations as compared to the normal Type III algorithm and the use of C rather than assembler.

A display of the current top ten finds was added to the program so that the slow progress of the search could be easily monitored. In addition a monitor of the current position in the database was shown. This addition, the loading of parameters from an external file and the ability to suppress display of alignments are present only in the PC version of the code.

"Fradho" does not use assembly level programming in its central routine. In the Turbo Pascal programs the assembly code was incorporated as hexadecimal numbers written directly into the program using the 'inline' directive. The assembler available for this work and the Turbo Pascal compiler were incompatible. Fortunately the optimised routines were small and so use of inline hexadecimal code was acceptable. No facility was available to add code 'inline' in the C programs. No attempt was made to rewrite the searching subroutine for "Fradho" in assembler.

In retrospect it was a mistake to attempt to write portable code from the start. The eventual need for assembler level code should have been apparent from the beginning. In any case, much of the code not actually in the main subroutine involves interactions with the machine's operating system and is machine dependent. Development of a Pascal version using the existing units would have tested the concepts far more rapidly and would have made it easy to use assembly level software in the main routine. Writing portable code meant designing for the lowest common denominator of the machines. The Sun code was encumbered by techniques to overcome memory restrictions on the PC and the PC code initially made limited use of the screen. A more productive approach would have been to develop code for the PC first and then subsequently a version for the Sun using the experience gained.

Variations in noise

A second kind of extension to the Type III algorithm was inspired by the recursive version of the algorithm used in "Medal". This extension was to tackle the problem of 'mixed noise'. The problem with a purely local homology algorithm is that it finds a single island of similarity. There may in fact be several such islands, and the combination of them gives far more evidence for the relationship. The variations in sequence between two proteins can be thought of as 'noise', the type of noise changing over the length of the proteins. In the core of the protein the noise would be expected to be low as changes in the core can seriously disrupt protein folding. In loop regions not involved with specific interactions the noise would be higher. The regions between the islands are regions of poor matching, that is regions of high noise.

It should be stressed that this new algorithm is a paper algorithm and has not been tested in practice. One reason for this is that the recursive algorithm in "Medal" already overcomes many of the problems of mixed noise and was used successfully to tackle the problem. A second reason is that the use of simultaneously run path matrices, an important algorithmic aspect of the new method, has already been demonstrated. The new algorithm would run two path matrices simultaneously. "Fradho" shows that the dynamic programming paradigm can be used successfully to switch between three simultaneously run path matrices to maximise an overall score. The new and untested method is described for several reasons. Firstly description of the method elaborates on a particular problem of the existing local methods. Secondly it shows a potentially more elegant solution to a problem than the one adopted in this work for "Medal". Thirdly the method is more suitable for parallelisation than the recursive method. Fourthly it demonstrates the scope still remaining within the dynamic programming paradigm for extension and enhancement to the basic algorithms.

Greedy and global optimisation

The recursive local alignment algorithm developed for "Medal" employs a 'greedy' optimisation algorithm. At each stage the algorithm picks the best remaining local similarity compatible with previous choices. Each choice may well exclude other choices. 'Greedy' algorithms generally find good but not optimal results. The greedy method does not guarantee to find the best combination of local similarities though it should generally find a good combination. A second defect in the recursive alignment algorithm is that it takes no account whatsoever of the quality of matching in regions between those which it has aligned.

Mixed noise Type III algorithm

A new algorithm for 'mixed noise type' could solve both these problems. The new algorithm finds regions that are globally optimal. The algorithm gives the highest scoring combination of high scoring islands, rather than picking the best island of matching available at each stage. It distinguishes between two kinds of noise and makes an automatic choice of scoring tables for different parts of the alignment. A natural choice would be between use of a 100 PAM table with a high indel penalty and 250 PAM table with low indel penalty for regions of higher noise.

The mixed noise algorithm calculates two path matrices simultaneously. The path matrices can be thought of as stacked one above the other. Additional path steps are possible between corresponding positions in the two path matrices. Traversing such steps incurs a penalty and corresponds to a change in noise type. The penalty is needed to limit switching between layers, just as an indel penalty limits the postulation of indels. The algorithm switches layers only if the net effect is to increase the score. Switching between the layers should incur a fairly heavy penalty. With no penalty for layer switching the path would simply use the least indel penalty and greatest score for diagonal steps of the two levels.

An additional minor benefit of this new method is that the path found through the pair of matrices not only yields an alignment but also indicates automatically which parts of the alignment are good and which parts of the alignment are poor.

One extra parameter is needed in addition to the layer switch penalty. The high noise regions of the alignment contain less information about sequence relationship per residue. It is appropriate to scale down the scores on the high PAM table by some factor. In low noise regions, matches would markedly increase the score and mismatches markedly decrease the score. In high noise regions neither matching nor mismatching would have much influence on the score. This scaling should cause the algorithm to use the low noise parameters to score regions which match well. The value for this scaling parameter would need to be determined by experiment. An extreme would be to score zero for all changes in the high noise part of the comparison. The recursive local alignment algorithm in "Medal" does something similar in not penalising or rewarding matches or gaps in regions between the matching islands of homology.

Comparison to recursive method

From experience of alignments performed by hand it is expected that in practice the mixed noise algorithm will not prove markedly superior to the recursive local similarity algorithm. Whilst it should do better on the poorly matching regions, it should at least make some attempt to align them, alignments of such regions are unlikely to be informative. In most cases the choice of islands of good matches made is not expected to be different from those made by the greedy strategy. Differences are, however, likely in the case of two proteins containing repeats of similar sequences. For such pairs, greedily choosing the best matching example of the repeated unit from each protein could preclude matching of some of the other examples. This would happen if the first example of a repeat in one protein matched best with the last example in another. In such cases the 'globally best' rather than 'greedily best' gives a better representation of the similarities. The suggested new algorithm is thus likely to be markedly better only for a relatively rare subset of comparisons.

Probably the most important advantage of the mixed noise algorithm is its greater suitability for parallelisation on a SIMD computer such as the DAP. The mixed noise algorithm requires a single sweep through the two path matrices. For the recursive algorithm multiple passes are required, which would make inefficient use of DAP hardware. Finding a dynamic programming analogue to the recursive algorithm gives an approach suitable for SIMD parallelisation.

Addendum: The "Blast" Algorithm

A recently published paper presents a new serial algorithm "Blast" that, though word based, overcomes the principle defects of the classical word based approaches (Altschul *et al.,* 1990).

The method allows inexact matching in its first stage of searching. It rapidly finds pairs of words of a fixed length W, one word from each sequence, that when scored using a Dayhoff table, score above a threshold T. The program then goes on to check in the neighbourhood of these words for further matching using the initial match as a seed. These further matches and mismatches are scored using the Dayhoff table. The method gives a rapid way to find high scoring word pairs of any length provided they contain a stretch of length W scoring greater than the threshold T. This searching strategy is linked to a semiempiric analysis of the likelihood of a good match with a particular score being missed, ie. the likelihood of a good match not containing a seed match scoring more than T.

The "Blast" strategy is also a flexible one. The authors have tried several variants in the neighbourhood searching once the seed has been located and they intend to adapt "Blast" to compare DNA against protein spotting frame shifts in the process. They say:

> "This permits the detection of distant protein homologies even in the face of common DNA sequencing errors (replacements and frame shifts)". (Altschul *et al.* 1990)

One adaptation of "Blast" searched for pairs of proteins with similarity to a query, on the principle that a three way alignment might reveal a pattern not obvious from a pairwise alignment (Altschul & Lipman, 1990). This was made possible by the high speed of their algorithm.

The savings that the basic "Blast" algorithm gives over examining every entry in the match matrix are impressive. "Blast" with its default wordsize of 4 and threshold of 14 need examine on average only 1 in 3200 positions. The fast Type III algorithm by contrast examines every single position.

"Blast's" method for finding word-pairs

The key feature of "Blast" is its method for finding word pairs scoring above the threshold. Prior to searching, the "Blast" method produces an expanded list consisting of all words that score T or greater against some word in the query. With default parameters W=4 and T=14 there are on average 50 words generated for each residue of the query. Rapid techniques are available and are used to identify exact matches of words from a database to words in the list. Using an expanded list is a way to convert the inexact matching problem to one of exact matching, as was achieved in a different fashion with alphabet reduction.

The default parameters give less sensitive matching than one might expect. With default parameters there is no guarantee of finding all matches of three residues in a run of four. This would require a minimum of 72 words in the expanded list per query sequence residue. In contrast, detection of exact dipeptide matches would guarantee to find all runs with three out of four matches. In this sense the "Blast" seeds are less sensitive than is exact dipeptide matching.

Increases in sensitivity can be obtained by reducing T or by increasing W; herein lies a major problem with "Blast", a problem which is noted by the authors. The list of words increases in size exponentially with decrease in threshold or increase in word size. Reducing T also leads to an exponential increase in running time as a higher proportion of matching words are found with a higher proportion of noise. Increasing W and T together could give greater sensitivity whilst maintaining or decreasing the proportion of noise matches. However, the exponential increase in list size precludes this method in practice. Thus in practice, sensitivity of seed matching cannot be markedly improved by changing the parameters.

The use of non-adjacent matching, as used by Roberts (1990) in multiple sequence alignment, could provide an alternative way to convert inexact matching to exact matching. Algorithmically a search for exact matching of four residues or more spread over seven residues can be made at one twentieth the speed for contiguous exact matching tetrapeptide searches. This is because there are twenty ways to spread four matches over a run of seven starting with a match. Possibilities for very high speed in tripeptide matching was shown in Chapter 5 so the method should be fast. The advantage of these seeds over "Blast" seeds would be the use of longer words, length seven, with a possible increase in seed sensitivity.

Validity of seed based matching

"Blast's" use of a seed match is open to the criticism that there is a non-zero probability of missing a good match with a high significance score. A good match need not contain a short high scoring seed segment. Since the significance score is only an approximate measure of biological significance, "Blast" provides an approximate method for obtaining an approximate measure, rather than an exact method for doing so. Given that the authors show that "Blast's" additional approximation is small, the criticism is less serious than might seem at first.

The total Type III comparison of the database performed in this work provides further motivation for an approach based on seeds, that is small regions of high scoring matching. In examining results from that study, clumping of matches gave greater confidence in the validity of an alignment. Every one of the sequence similarities reported in this thesis contains a run with three out of four residues matching. The approach of using a seed and extending alignments from it is therefore a promising method for high performance database searching.

Appendix 1: Tests of some Ideas for New Software

Introduction

Problems with existing software for biological sequence work motivated some investigation of alternative methods. Approaches to three particular problems are described. The first two problems are ones commonly faced by molecular biologists. The third is a specific problem of pattern identification.

Sequence Retrieval

Background:

A frequent requirement of molecular biologists is to retrieve sequences from sequence databases by name. Even if the code name is available there may be slight problems. For example, different databases use different code names for the same sequence. Often code names for the sequences sought are not available and the researcher has only a descriptive sequence name. Alternatives in nomenclature or use of abbreviations cause problems with retrieval strategies that use keywords. Keyword matching, as used in the GCG package's "Strings" and the 'find' commands of "PSQ" and "PDQ" fail to retrieve 'Trp synthase' when asked for 'Tryptophan synthetase'. GCG's "Strings" is also notable for taking a long time to scan for text matches. "Strings" does not use indices, instead it checks each sequence title line in turn for a match. This is slow and discourages inquiries about alternatives. The more efficient "PSQ" and "PDQ" which do use indices are on the other hand offputting to users. The national Molecular Biology computing centre at Daresbury which supports all three packages has found that most users use the GCG "Strings" program in preference to "PSQ" and "PDQ" in spite of encouragement to use the more efficient programs.

Idea test:

'Key word in context' indices are familiar to molecular biologists. They are used, for example, for indexing titles of papers in 'Biological Abstracts'. The index is a list of one line entries. Each entry occurs several times, once for each significant word, and the occurrences are sorted on these words. A sample is shown below:

```
/NEPHROPATHOLOGY OF CYSTIC FIBROSIS A HUMAN MODEL OF
  LUNG INFECTION IN CYSTIC FIBROSIS A LONGITUDINAL ST
 CHLORIDE LEVELS IN CYSTIC FIBROSIS A NEGATIVE REPORT
```

An entry may occur elsewhere in the printed index, as follows:

```
   OF STREPTOCOCCAL INFECTION IN CULTURED YELLOWTAILS
  S-AURUGINOSA LUNG INFECTION IN CYSTIC FIBROSIS A LO
  HYLOCOCCUS-AUREUS INFECTION IN CYSTIC FIBROSIS HUMA
```

An online keyword in context index of the protein title lines was made. Due to its size, the individual words were tokenised, keywords being replaced internally by two byte tokens. This resulted in 9182 tokens, each protein title generating on average 8.3 tokens. To the user the index appeared as a normal file of 67,280 lines which could be examined on the PC using a simple interface program.

The formation of the index necessary for tokenisation uncovered many misspellings in the database, e.g oncogene with zero for an 'o' and 'falvoprotein' for 'flavoprotein'. The extent of inconsistent nomenclature and of typing mistakes in the databases has been commented on elsewhere (Tullo & Attimonelli, 1989). The samples given here defeat the keyword in context strategy. For other mistaken entries such as 'antennepedia' for 'antennapedia', the keyword in context index does help. Keyword methods alone would fail in cases like this one.

Comments:

Although the keyword in context strategy does not automatically match up alternative nomenclature, its rapidity and ease of use do make it easy for biologists to check alternative names. Also some near misses (e.g 'synthase' and 'synthetase') are drawn to the biologist's attention.

A full solution to the problems of variation in nomenclature and typing mistakes in the database must rest with the database administrators. It is their task to standardise nomenclature or alternatively to provide indices of equivalent names. They too are in a position to organise the non-sequence data for well developed commercial free text retrieval systems.

Dotplots

Background:

A graphical method for displaying similarities between pairs of sequences is the 'dotplot'. As in the path matrix (Chapter 2) one sequence is placed horizontally and the other vertically. At its simplest a dot is placed at positions where the two sequences agree. Runs of similarity between the two sequences appear as diagonal lines on the dotplot. For DNA sequences and to a lesser extent protein sequences, background noise from random matching tends to obscure these line. A system of filtering is usually employed. This uses two parameters, a windowsize and a threshold. In the filtered dotplot, dots are removed unless they occur in some diagonal segment of length windowsize that has more than the threshold number of matches, i.e. only dots in regions with a high density of matching are shown.

Dotplots are time consuming to produce. With the GCG package two programs need to be run. One generates a list of points, the other displays them. This very strongly discourages experimentation with different parameters. Unfortunately experience and patience seem to be necessary to get the clearest dotplots.

A subsidiary problem with dotplots is that detail in a dotplot can be hard to see as the axes may be compressed to fit the dotplot on the screen.

Idea test:

A program was written to implement scrolling of graphical images on the IBM PC. Instead of displaying compressed dotplots, only as much of the dotplot as would fit on the screen at the most detailed resolution was shown. The software written to scroll the image permitted viewing of other parts. For speed this software copied already calculated parts of the image where possible so requiring a minimum of recalculation.

Other aspects of the implementation addressed the problem of speed in calculation. Segments which overlap can share some of their counting. For speed the counting was organised to exploit this and indices were used to determine which counts to increment. Also dots were plotted in colours depending on the number of matches in the window. This enabled very rapid selection of different thresholds once the dotplot was complete. This was achieved by manipulating the colour palette of the computer without otherwise changing the data stored in the graphic memory. Scores below the current threshold could be given the background colour so making only dots with scores above the threshold visible. Changing threshold was virtually instantaneous.

Comments:

With the test software, scrolling was slow even though this used the built in graphics memory copy commands. Unlike some microcomputers, access to graphical memory on the IBM PC is indirect. This markedly slows graphics operations. For IBM PCs speed can best be achieved by minimising the number of changes to the graphic image. Consequently a high level view with a zoom option is probably a better way to achieve detailed viewing of any part than the method described here.

The GCG implementation suffers from slow graphics which partially explains its slow operation. However, its main loss in speed is due to splitting the task into two stages and the use of an intermediate points file. Writing point information to the file and then reading it back in, as the GCG implementation does, is unnecessary and wasteful.

The methods discussed here make testing different parameters more rapid. With two parameters there are still many combinations to try for any one pair of sequences. If some method could be found to remove one parameter, leaving only a 'plot density' parameter, this might provide a more satisfactory solution.

Identification of a target pattern

Background:

Type I restriction enzymes cut DNA sequences containing specific patterns. Unlike Type II enzymes they do not cut at the recognition site but instead cut at a variable distance from the target sequence. Patterns recognised by three Type I restriction enzymes STySP, EcoK and CfrA are:

STySP:	gag n_6 rtayg
EcoK:	aac n_6 gtgc
CfrA:	gca n_8 gtgg

Here n represents any residue the subscript showing how many, and y stands for either c or t, r for g or a.

A new Type I restriction enzyme, EcoE, had been purified and tested on a number of sequences by other researchers. It was desired to find out what DNA pattern it recognised. A list of sequences cut and those not cut was available.

Idea test:

An approach to the pattern identification problem that uses existing software is to produce a list of overlapping DNA fragments of length 16 from the sequence data and to sort this list on two keys. The first key would have 3 characters, the second 4. The keys would be separated by a distance of between 4 and 9 residues. These lists, one for each distance, could then be printed and manually scanned. Groups of fragments that agreed in the selected positions would occur together in the list. Such groups containing fragments from the cut sequences and no fragments from the uncut sequences would indicate possible target patterns.

A program was written that was an interactive implementation of this process. Sorting was performed on any base position and the sorted list examined on the screen. A stable sort was used so that repeated sortings could produce any required multi-key sort. Next fragment groups were defined by selecting base positions in the fragments. Fragments adjacent in the list agreeing at these positions were grouped together. Rather than manually scanning for groups that were compatible with the experimental results, sorting was used again in the following manner. Each fragment of each group was tagged with an indication of the sequences, cut and uncut, in its group. That is, an extra field was introduced to represent this information. A stable sort on this tag field brought sequence groups with the same cut and uncut sequences together. It was then easy to find groups with fragments from all cut sequences and none from uncut sequences. The program identified the following pattern as a possible target:

EcoE: gag n_7 atgc

And showed that no other pattern with a structure similar to known recognition patterns for Type I enzymes was consistent with the data (Cowan *et al.* 1989). The following near miss patterns, it was noted, were in sequences not cut by EcoE:

```
HsdK:    aag gaagaga atgc
pBR322:  ggg catcccg atgc
M13:     gaa ttacctt atgc
pBR322:  gag cgagggc gtgc
HsdSP:   gag ttgttcg acgc
M13:     gag tacggtg atac
M13:     gag cgtcaaa atgt
```

The tool acted as a way to rapidly organise information that had been collected by the researchers. Organising the fragment results identified the target pattern.

Comments:

The grouping operation was an important feature of the program and not available as an adjunct to existing sorting programs. In hindsight the software could have been made more automatic and would still have given the correct answer. Use of existing software was tried initially. Whilst simplifying the task it would have required considerably more work on the part of the researcher than the approach described here.

Appendix 2: Methods for Fast Serial Type III Comparison

The Type III algorithm can be described economically as follows: A symmetrical table that scores similarities of pairs of characters from an alphabet is used to generate scores d[i,j] for the similarity of the ith character of one string to the jth character of the second. Positive d[i,j] indicate similarity, negative d[i,j] are counterindicative of similarity. A penalty P, the 'indel' penalty, represents the cost of inserting a gap in either sequence. Using these elementary scores, a score S[i,j] for the best local similarity ending on the ith character of the first sequence and jth character of the second can be computed. The score S[i,j] is expressed recursively as:

```
S[i,j] := Max( S[i-1,j-1]+d[i,j],
               S[i,j-1]-P,
               S[i-1,j]-P,
               0 )
```

This expression is for $0<i\leq m$ and $0<j\leq n$. S[i,0] and S[0,j] are equal to 0 for $0\leq i\leq m$, $0\leq j\leq n$ respectively.

Matching the ith character against the jth corresponds to the first term and increases the score S at [i,j] by d[i,j] over its value at S[i-1,j-1]. The second and third terms correspond to the cost of skipping a character in either sequence. This is -P. The formula for S[i,j] calculates the best similarity for substrings ending at characters i and j as being from matching, skipping one or other character, or if all three give negative results, starting a run of similarity after this position. The largest value of S[i,j] gives the score for the best local region of similarity between the two strings.

In a recursive implementation to calculate the S[i,j] there would be wasteful recalculation of previously calculated values. Instead the dynamic programming technique is used and all results $0<i\leq m$, $0<j\leq n$ are computed in a suitable order. Computation time is thus *O(mn)*.

Re-expression

The majority of pairs of strings compared in the Molecular Biology application have low similarity and most of the S[i,j] are less than P. To use this observation to increase efficiency requires some rearrangement to the equations. The intermediate stages of rearrangement result in code which is manifestly less efficient.

Taking the original formulation we first increase indices. For 0≤i<m and 0≤j<n we have:

```
    S[i+1,j+1] := Max(  d[i+1,j+1]+S[i,j],
                        S[i+1,j]-P,
                        S[i,j+1]-P,
                        0)
```

We can introduce extra tests and break the 'Max' into components.

```
S[i+1,j+1] := Max(0,d[i+1,j+1])
if S[i,j]   > 0 then
      S[i+1,j+1] := Max(0,d[i+1,j+1]+S[i,j])
if S[i+1,j] > P then
      S[i+1,j+1] := Max(S[i+1,j+1],S[i+1,j]-P)
if S[i,j+1] > P then
      S[i+1,j+1] := Max(S[i+1,j+1],S[i,j+1]-P)
```

The same positive values can be obtained as follows:

```
T[i+1,j+1] := d[i+1,j+1]
if T[i,j]   > 0 then
      T[i+1,j+1] := T[i+1,j+1]+T[i,j]
if T[i+1,j] > P then
      T[i+1,j+1] := Max(T[i+1,j+1],T[i+1,j]-P)
if T[i,j+1] > P then
      T[i+1,j+1] := Max(T[i+1,j+1],T[i,j+1]-P)
```

This change modifies the treatment of zero. Unlike S[i,j], T[i,j] may become negative. S would hold a zero in such positions. S and T however agree on their positive values. A negative T[i,j] fails each of the comparison test and behaves just as if it were zero in so far as its influences on other T[i,j] is concerned. That S and T agree on positive values can also be checked by considering the cases leading to T[i+1,j+1]≤0 and T[i+1,j+1]>0 separately. Because S and T agree on positive values they find the same similar substrings.

Rearrangement

This new form permits a rearrangement. Before the main loop starts all the T[i,j] are initialised to d[i,j]. In the rearranged loop instead of collecting values for one array element, values are distributed from an element that has positive score. The operations performed at each location in T are now as follows:

```
Start     if T[i,j]>0 then begin
             if T[i,j]>P then begin
                T[i+1,j] := Max(T[i+1,j],T[i,j]-P)
                T[i,j+1] := Max(T[i,j+1],T[i,j]-P)
             end
             T[i+1,j+1] := T[i+1,j+1]+T[i,j]
          end
```

Testing for T[i,j]>P is necessary only if the testing for T[i,j]>0 has already succeeded. The testing substantially increases the speed. In a typical comparison of a pair of sequences around three quarters of the T[i,j] are negative. These are skipped. This is where the main saving from the rearrangement arises. Of the remaining values around one half score P or less and are dealt with rapidly. The second comparison requires subtraction of P from T[i,j]. The result of this subtraction is then used twice if the test succeeds. The original formulation in terms of S required two subtractions of P per calculation of S[i,j] so there is an overall saving in calculation even when the second test succeeds.

Use of registers

The next version of the pseudocode shows minor changes to take account of the possibility of using registers. To achieve greater speed the algorithm was implemented at assembly level. A word length register AX was used to hold the current T[i,j] and the register CX to hold T[i,j] - P. At this point the influence of the target processor, the 80286 may be clear. The choice of processor is primarily a reflection of the ready availability and affordability of IBM PCs and compatibles. Use of registers to improve speed is applicable to most processors.

A standard technique to reduce storage and indexing calculations for the matrix T was also used. Only two of T's columns are needed during processing.

T0[j] takes the place of T[i,j] and T1[j] the place of T[i+1,j]. After processing one column, the values in T0 are no longer required. T1 is used in place of T0 and the old T0 is initialised with the appropriate d[i,j] values and takes the place of T1. The new inner loop is shown below.

```
Start:    AX := T0[j]
          if AX>0 then begin
             CX := AX-P
             if CX>0 then begin
                T1[j]   := Max(T1[j],CX)
                T0[j+1] := Max(T0[j+1],CX)
             end
             T1[j+1] := T1[j+1]+AX
          end
          j := j+1
          if j<= n then goto start
          stop
```

Deferred assignment.

A technique compilers use to improve efficiency in code they produce is to defer assignments (Appendix 3). A compiler may defer the writing of a value held in a register back out to memory. This can lead to a saving when the same memory location is written to several times in succession. This situation frequently occurs in executing instructions in a loop. A memory location written to in one cycle of the loop may be written to again in the subsequent cycle. This algorithm illustrates potential for a more complex form of deferred assignment, conditional deferred assignment. In the unmodified code writing to memory locations is conditional on values calculated within the loop. Depending on the condition, the body of the loop may or may not write a new value out to memory. To cope with conditional deferred assignment, different versions of the loop were required. These handle different states of deferred assignment from the previous execution of the loop. In this code, the 'code expansion' was used to allow deferral of assignment to T0[j+1] and T1[j+1]. If CX>0 then both deferrals take place, if only AX>0 then only deferral of assignment to T1[j+1] takes place, and if AX≤0 then no deferred assignment is required. This required three versions of the loop body. As each version completes a cycle, it enters the appropriate version for the next cycle. Which version it enters will depend on which assignments are being deferred.

In fact the value being held to write into T0 need not be written at all. The value is needed only in the next cycle round the loop. For this use it can be taken from the register holding the value that is being deferred. Use in determining maximum score is the only other potential subsequent use for values written into T0. Since the deferred value is derived by subtracting P from a value already in the matrix T, it cannot itself be the maximum score. The result does not need to be written out to memory. We now have:

```
Start1:     AX := T0[j]
            if AX>0 then begin
               CX := AX-P
               if CX>0 then begin
                  T1[j] := Max(T1[j],CX)
                  goto DeferTwo
               end
               goto DeferOne
            end
NoDefer:    j := j+1        ;AX<=0
            if j<= n then goto Start1
            Stop

Start2:     DX := AX+T1[j]
            AX := T0[j]
            if AX>0 then goto Morecalc
            T1[j] := DX
            goto NoDefer
DeferOne:   j := j+1        ;AX>0, CX<=0
            if j<= n then goto Start2
            stop

Start3:     DX := AX+T1[j]
            AX := T0[j]
            AX := Max(AX,CX)
;No need to test for AX>0 here as AX>=CX>0
Morecalc:   CX := AX-P
            if CX>0 then begin
Maxtest:       T1[j] := Max(DX,CX)
               goto DeferTwo
            end else begin
               T1[j] := DX
            end
            goto NoDefer
DeferTwo:   j := j+1        ;AX and CX>0
            if j<= n then goto Start3
            stop
```

The number of external memory references has been decreased. The plethora of gotos is not a concern. The unconditional gotos can be removed by rearranging the code and code duplication. Other unconditional gotos originating from expanding 'if then else' and 'Max' can be removed in a similar fashion.

Further code expansion is possible. In the line AX := Max(AX,CX) the conditional assignment of CX into AX could be replaced by a conditional branch to equivalent code with the roles of CX and AX reversed. This however was not done. It would nearly double the length of the program for a marginal performance gain.

Maximum value and rogue value

Determination of the overall maximum value in the array and its position has so far been left out of this discussion. The maximum value in a column could be determined by a second loop that scans every entry in the column. In fact, since we already have a loop which picks up values from T0 we can be far more efficient about finding the maximum. The overheads for looping and fetching values from T0 can be dispensed with by building the maximum testing into the loop. Moreover we only need the test in the version of the code which performs both deferrals, since deferrals correspond to values in T0 greater than P. This reduces the number of times we test for the current maximum being exceeded by a factor of six. In doing this we have lost the ability to correctly score low scoring pairs. The code will only detect those scores greater than P. For the database searching application this is no loss. Substantially higher scores are needed for evidence of relatedness between compared proteins. An even better position for the maximum test is at the line labelled maxtest, when the value DX has been found to be greater than CX. Any matching of two residues where the score for the substrings before matching the two residues is greater than 2P will reach this point. This gives a slightly higher threshold for guaranteed reporting of scores and an even less frequent execution of the maximum test.

Loop overheads

The loop overheads, the test for $j \leq n$, are now a significant fraction of the algorithm's cost. An improvement that is possible since the test for maximum is infrequent is the use of a rogue value for determining whether the loop has completed. T0[n+1] is initialised with an impossibly high value. This causes entry into the code which checks to see if the current maximum score has been exceeded. If it has, this code additionally checks for the rogue value. Using a rogue value in this way virtually eliminates the time spent in checking for the end of the loop. The tests for $j \leq n$ can be replaced with unconditional gotos. The testing of AX>0 in the most commonly executed case, no deferral, is a natural candidate for 'loop unrolling'. This unrolling makes a saving since it reverses the test so that the conditional branches most commonly do not branch.

At the start of each cycle it is necessary to initialise the vector T1. This initialisation is dependent on the ith character of the second sequence. This is most rapidly performed if vectors for each of the characters in the alphabet are precomputed. Initialisation is then performed using a rapid memory copy operation.

Performance

The overall result of these optimisations is an algorithm which has a speed of 300,000 PMEs^{-1} on a 16 MHz IBM PC. This compares to speeds implied by Pearson (1990) of 4,000 PMEs^{-1} for comparison; and by Mount (1988) of 700 PMEs^{-1} for comparison and alignment on a mainframe and a speed of 6000 PMEs^{-1} for Pascal code on the 16 MHz IBM PC (own data). Thus the optimisations described here lead to an approximate 50 fold speed improvement.

Appendix 3: Conditional Deferred Assignment

Just as improvements can be made in disk performance by disk caching, that is, keeping of partial copies of disk files in RAM memory, so too speed improvements can be made by keeping copies of some RAM memory locations in the processor registers. In both optimisations multiple reads or writes to the slower memory are replaced by multiple reads and writes to the faster memory. Temporary use of processor registers to hold a copy of a RAM memory location is an important optimisation technique. Aho *et al.* (1986) discuss how a compiler can automatically select which memory locations to hold in a register. Any values changed in the faster memory are ultimately written from the fast memory to the slower. It is deferral of this process of assigning a value held in a register to a variable held in RAM memory that leads to savings when multiple writes to slow memory would normally be involved. Deferral allows one assignment to slow memory to take the place of several.

With disk caching, the timescales are such that decisions about when to write out changed data can be made at run time by software. These decisions can depend on current availability of memory to hold disk data. With deferred assignment, the decisions are made at compile time. The point in the software at which to write register values to memory must be decided in advance and built into the program. To do this, as the software is compiled a history of which reads and writes to memory occur must be built up.

Information about the history of memory reads and writes can be updated incrementally with each line of code compiled. Conditional branches in the code can lead to alternative histories. The usual solution to this is, where two code streams meet, to use only the history common to both branches that meet. This can lead to a loss of history information and the loss of the ability to defer assignment. In particular it leads to the loss of ability to defer a conditional assignment.

The transformation to recover the property of having a known history is straightforward. Where two branches of the code merge they may do so in the transformed code only if the history, in as far as it affects deferred assignment, is the same. Otherwise a copy of the relevant code is made.

In the program in Appendix 2, two memory locations are candidates for deferral, leading to four possible code variants. In fact, update of one of the memory locations is conditional on update of the other, so only three variants are actually required. This code expansion effectively remembers alternative histories. This extends the optimisation technique of deferred assignment to the case where some of the operations during the deferral are conditional.

Appendix 4: Potential for Optimisations to "Prosrch"

The program "Prosrch" (Lyall, 1988) performs a rapid Type III search, single sequence against database on the DAP parallel computer. Most of the code in the program concerns user interface and file I/O. Here we are concerned only with the time critical core comparison routine which represents less than one tenth of the total code. Before describing possible changes, we describe the implementation as it currently stands.

"Prosrch" keeps two rows of the comparison matrix at any time. These rows are updated in a parallel fashion. Rows may have up to 32768 elements and contains several sequences from the database. Sequences are delimited by sequence separator characters which are used to prevent scores from one sequence influencing scores in another.

The row elements carry three pieces of information, the score, the starting coordinate in the path matrix of the path leading to that score and the best score so far on this path. Whenever the score falls below zero this information is reset. The best score is set to zero and the coordinate information is reinitialised to the current row and column. The program keeps a global threshold value. Scores are tested against this threshold after each update of the rows. Scores on paths which have just taken a positive step and are above this threshold and also above their record of the best score so far are dealt with in a serial subroutine called 'Dsavres'. These scores and their associated coordinate information are recorded serially in a separate array. The code in 'Dsavres' has to identify whether a score has already been reported for this path's starting coordinate. If so it locates where its top score is held; if not it allocates a location.

Ideally the threshold would be set so that exactly the required number of results, typically 16,000, would be collected. In practice the distribution of scores is not known exactly in advance. This being so, the threshold is set to a reasonable but low value and is increased as results are collected. Dsavres is called many times more than the number of results collected. On a good path, each increase in score could lead to a call to Dsavres. These calls are additional to calls early on before the threshold has approached its ultimate level. One approach developed by J.F. Collins (ICMB Edinburgh, personal communication) that has considerably improved the performance is the development of superior techniques to set and adjust the threshold.

Optimisation

Given that fast serial implementations for Type III searching are possible, it is natural to explore the potential for optimising the parallel code. The changes to "Prosrch" presented here are changes which show the potential for optimisation but which have not been tested in practice. To do so would require changes in the interface and post-processing code in addition to changes made in the core code.

For the modified core, written in DAP FORTRAN, use was made of the C language's pre-processor, "cpp". This was so that repeated definitions of datastructures, repeated definitions which are required by FORTRAN, were generated accurately and automatically. Blocks defining the use of memory were placed in a single file. The C pre-processor included selected portions of this file into the source code in the appropriate places. This change facilitates modification of the datastructures, the use of subroutines and it also simplifies the appearance of the "Prosrch" program.

The post search phase of "Prosrch" runs on a serial machine and processes fewer than one in a hundred of the proteins examined in the search phase. The first step taken in optimisation was to fully exploit savings that could be made by shifting work to the post-search phase. In fact, in searching, all that each row element needs to carry is the score. With this modification the program would produce a single score for each protein, the score of its best match. The searching program would not determine the coordinates in the path matrix of the ends of the best path, leaving this to the post-search phase. The scores determined in this modified search could be used exactly as before to produce the same list of high scoring proteins as produced by the current "Prosrch" program.

An important loss associated with this scheme is loss of results additional to the best similarity for each protein in the database. "Prosrch" collects more than one result per protein. The bulk of these are chance matches of unrelated proteins. The distribution of the scores of these is used by "Prosrch" to measure the likelihood that a high scoring match is part of the noise distribution. The Dayhoff scores for an alignment are converted to likelihoods using an exponential transform based on the collected distribution.

The Dayhoff scores themselves give a logarithmic measures of likelihood. The computation of likelihoods using the distribution is a refinement that has not yet been proven to give more useful information. The order in which results are presented by "Prosrch" depends only on the score. The computation using the additional noise results does not affect the order of results.

The extra results do have a second more important function as they can give additional information about relatedness when a protein has more than one region of matching. In current implementations of the post-processing phase of "Prosrch" these extra results are not used to affect the order in which results are reported. If a protein contains two weakly matching regions, its position in the list of results is determined by the highest scoring one of these weak matches.

With the suggested alternative method, if detection of additional regions of matching for each reported protein is desired then the reported proteins could be reexamined using a serial or a parallel approach. Whether a serial or parallel approach is used, the workload of this stage would be less than one hundredth of the workload of doing this for all proteins in the database.

The changes to record one score per protein reduce the quantity of information being transferred between processor elements by a factor of four and should increase execution speed by at least the same factor. This factor can be increased to eight by using only one byte to hold scores rather than two bytes. Scores for comparisons scoring more than 256 would overflow and the true score would not be reported. The highest score achieved before overflow would be reported instead. Re-analysis of the small number of high scoring matches would reveal the true situation.

Details

In the interests of clarity a number of finer points have been left out of the discussion. The "Prosrch" program makes extensive use of a DAP specific speed improving technique called crinkling. With crinkling, a single processor holds several consecutive row elements. Fortunately the new technique can crinkle in much the same way as "Prosrch" crinkles. Extra separator characters can be introduced between sequences to ensure that each DAP processor element deals with only one sequence. Best results can then be collected on a per-processor rather than on a per-element basis. This leads to slight savings, most notably reducing the storage required for best results by a factor of the number of row elements held per processor.

The overflow of scores also requires careful consideration. In fact, since the score must on occasion hold small negative values, the byte values should be considered as covering the range -64 to +191 rather than 0 to 255. This does not affect the standard DAP arithmetic, an observation which needs careful checking, but this asymmetric range does affect the criterion used in detecting negative scores to reset scores to zero and requires a slight modification to the test.

Effect

Even with these complications the changes produce a dramatic simplification to the searching code. The core part of the routine shrinks from 250 lines to 100 lines. The subroutine Dsavres containing 400 lines of code is replaced by the single line:

```
PSCORE(N) = MAXV( MSCORE , PROT.EQ.N )
```

to record the maximum score for a protein. This statement is executed once per protein, once for each value of N, whereas Dsavres is called for every improvement to a path that scores above the threshold.

The simplifications are also important for future development. The more economical code should be easier to develop to use the more complex dynamic programming comparison techniques of Chapter 9. The mixed-noise algorithm described there, which returns a result dependent on all islands matched, could be used to provide a parallel counterpart of the serial recursive alignment algorithm used in "Medal".

Further comment

These techniques have been discussed with J.F. Collins who has suggested further simplifications. He also suggested that a better estimate of significance could be obtained using both score and alignment length rather than score alone. This estimate could be based on statistics collected with the existing "Prosrch" program.

The first additional simplification he suggested was use of the range -128 to +127 rather than -64 to +191. This simplifies the arithmetic tests. Secondly, rather than producing one score per protein, finding maximum scores for a block of typically ten proteins has some advantages. Since the databases are too large to be processed in one section, the "Prosrch" program groups proteins into blocks. Rather than detecting high scoring proteins, the modifications could be used to detect high scoring blocks instead. The new software would then act as a fast first stage filter for the existing comparison and alignment software. This 'block filter' would reject blocks consisting entirely of proteins with similarity to the query scoring below some threshold. Unlike the two stages of "FastP", the filter and more sensitive stages would be compatible. All proteins with alignments scoring highly by the criteria of the second stage would be guaranteed to be accepted by the first stage. The most important advantage of this approach is that far less modification to the existing post-processing software would be required.

Abbreviations

Computer Science and Molecular Biology abbreviations:

ATP	adenosine triphosphate.
AMP	adenosine monophosphate.
cAMP	cyclic adenosine monophosphate.
DNA	deoxyribonucleic acid.
EGF	epidermal growth factor.
I/O	input/output.
MIMD	multiple instruction multiple datapath.
NADH	nicotinamide adenine dinucleotide.
NMR	nuclear magnetic resonance.
NWS	Needleman Wunsch Sellers.
PAD	packet acceptor/distributor.
$PMEs^{-1}$	path matrix elements per second.
PR	pathogenesis related.
PTS	phosphotransferase system.
RAM	random access memory.
RNA	ribonucleic acid.
SIMD	single instruction multiple datapath.
tRNA	transfer ribonucleic acid.
YP	yolk protein.

Organisation and product names and abbreviations:

AMT	Active Memory Technology limited (Reading U.K.).
DAP	Distributed Array Processor (parallel computer manufactured by AMT).
EV/PSX	Mark name of Evans and Sutherland limited.
GCG	Genetics Computer Group (Devereux *et al.,* 1984).
IBM	International Business Machines limited.
ICL	International Computers limited.
ICMB	Institute of Cell and Molecular Biology (Edinburgh University).
NBRF	National Biomedical Research Foundation.
PC	personal computer (usually IBM PC).
PIR	Protein Information Resource (name of the NBRF protein database).
Sun	Mark name of Sun systems limited.
Turbo Pascal	Registered trademark of Borland International Inc (Scotts Valley, CA 95066-9987).
VAX	Mark name of Digital computers limited.
VAX/VMS	Mark name of Digital computers limited.

Selected programs, description and references:

"Blast"	-- Fast sequence comparison using high scoring word matches as seeds (Altschul *et al.*, 1990).
"FastA"	-- Fast sequence comparison using exactly matching words (Pearson, 1990).
"O"	-- Program for displaying three dimensional protein structures (Jones T.A., Dpt. Chemistry, Aarhus University, Denmark).
"PKZip"	-- Data compression (PK-WAre ltd, WI 53217, U.S.A.).
"Prosrch"	-- Database searching using Type III algorithm on a parallel computer (Lyall *et al.*, 1986).
"PSQ"	-- Protein sequence entry retrieval (NBRF).
"PDQ"	-- Protein sequence entry retrieval (UIG, Daresbury laboratory, Warrington U.K.).
"Strings"	-- Protein sequence retrieval by name (GCG).
"Wordsearch"	-- Exact word matching database searching (GCG).

Selected own programs, description:

"Dayhoff"	-- Generation of dayhoff scoring tables.
"Fradho"	-- DNA to protein comparison.
"Medal"	-- Multiple sequence editor and aligner.
"Prowl"	-- Database searching using Type III algorithm on PC.
"Xref"	-- Annotation file crossreference browser.

References

Aho A.V., Hopcroft J.E. & Ullman J.D. (1983) "Data structures and algorithms" p.377 Ex. 11.23, Reading MA: Addison-Wesley.

Aho A.V., Sethi R. & Ullman J.D. (1986) "Compilers: Principles, techniques and tools" pp.517-522, Reading MA: Addison-Wesley.

Aiba H., Mori K., Tanaka M., Ooi T., Roy A. & Danchin A. (1984) "The complete nucleotide sequence of the adenylate cyclase gene of Escherichia coli" *Nucleic. Acids Res.,* **12(24):** 9427-9437.

Akyoshi D.E., Regier D.A., Jen G. & Gordon M.P. (1985) "Cloning and nucleotide sequence of the tsz gene from Agrobacterium tumefaciens strain T37" *Nucleic. Acids Res.,* **13(8):** 2773-2788.

Altschul S.F., Gisch W., Miller W.E., Myers E.W. &Lipman D.J. (1990) "Basic local alignment search tool" *J. Mol. Biol.,* **215 (3):** 403-410.

Altschul S.F. & Lipman D.J. (1990) "Protein database searches for multiple alignments" *Proc. Natl. Acad. Sci. USA,* **87(14):** 5509-5513.

An F.Y. & Clewell D.B. (1991) "Tn917 transposase. Sequence correction reveals a single open reading frame corresponding to the tnpA determinant of Tn3-family elements" *Plasmid,* **25(2):** 121-124.

Arfken G.B. (1985) "Mathematical methods for physicists" 3rd edn. Orlando: Academic Press

Atkins J.F., Weiss R.B. & Gesteland R.F. (1990) "Ribosome gymnastics: Degree of difficulty 9.5 Style 10.0" *Cell,* **62(3):** 413-423.

Bains W. (1986) "Multan - A program to align multiple DNA sequences" *Nucleic. Acids Res.,* **14(1):** 159-177.

Bairoch A. (1989a) "PROSITE: A dictionary of protein sites and patterns" 4th edn. University of Geneva.

Bairoch A. (1989b) "PROSITE: A dictionary of protein sites and patterns" 4th edn. p.53 University of Geneva.

Barker W.C., George D.G. & Hunt L.T. (1990) "The protein sequence database" *Methods in Enzymol.,* **183:** 31-49.

Barton G.J. & Sternberg M.J.E. (1987) "A strategy for the rapid multiple alignment of protein sequences" *J. Mol. Biol.,* **198:** 327-337.

Bollobas B.J. (1979) "Graph theory: An introductory course" New York: Springer Verlag.

Bradshaw A. (1987) "The control of cell growth: The role of polypeptide growth factors and oncogene products." in "Oncogenes and growth factors", Bradshaw R.A. & Prentis S. eds. pp.x-xv, Amsterdam: Elsevier.

Brennan R.G., Weaver L.H. & Matthews B.W. (1986) "Use of protein sequence and structure to infer distant evolutionary relationships" *Chemica scripta,* **26B:** 251-255.

Burks C. *et al.* (1990) "Genbank: Current status and future directions" *Methods in Enzymol.,* **183:** 3-22.

Cabot E.L. & Beckenback A.T. (1989) "Simultaneous editing of multiple nucleic and protein sequences with ESEE" *CABIOS,* **5:** 233-234.

Claverie J.M. & Sauvaget I. (1985) "PGtrans: A protein sequence databank generated by computer translation of Genbank nucleotide sequences" *Trends Biochem. Sci.,* **10:** 8.

Claverie J.M. & Bougueleret C. (1986) "Heuristic informational analysis of sequences" *Nucleic. Acids Res.,* **14(1):** 179-196.

Collins J.F. & Coulson A.F.W. (1987) "Molecular sequence comparison and alignment" in "Nucleic acid and protein sequence analysis: A practical approach" Bishop M.J & Rawlings C.J. eds. Ch13 pp335-336, Oxford: IRL Press.

Cowan G.M., Gann A.A.F. & Murray N.E. (1989) "Conservation of complete DNA recognition domains between families of restriction enzymes" *Cell,* **56:** 103-109.

Dayhoff M.O., Schwartz R.M. & Orcutt B.C. (1978) "A model of evolutionary change in proteins" in "Atlas of protein sequence and structure" Vol.5 Supp.3 Ch.22 pp345-352, National Biomedical Research Foundation.

Debelle F. & Sharma S.B. (1986) "Nucleotide sequence of the Rhizobium meliloti RCR2011 genes involved in host specificity of nodulation" *Nucleic. Acids Res.,* **14:** 7453-7472.

Devereux J., Haeberli P. & Smithies O. (1984) "A comprehensive set of sequence analysis programs for the VAX." *Nucleic. Acids Res.,* **12(1):** 387-395.

Devereux J., Haeberli P. & Marquess P. (1989a) 'Bestfit' in Program manual section of GCG package user manual, Sect.5 p.17. Madison: University of Wisconsin Biotechnology Center.

Devereux J., Haeberli P. & Marquess P. (1989b) 'Gap' in Program manual section of GCG package user manual, Sect.5 pp.25-31, Madison: University of Wisconsin Biotechnology Center.

Doolittle R.F. (1981) "Similar amino acid sequences: Chance or common ancestry?" *Science,* **214:** 149-159.

Doolittle R.F., Hunkapiller M.W., Hood L.E., Devare S.G., Robbins K.C., Aaronson S.A. & Antoniades H.G. (1983) "Simian sarcoma virus onc gene, V-sis is derived from the gene (or genes) encoding a platelet-derived growth factor" *Science,* **221:** 275-276.

Doolittle R.F. (1990) "Searching through sequence databases" *Methods in Enzymol.,* **183:** 31-49.

Efstratiadis A., Posakony J.W., Maniatis T., Laun R., O'Connel C., Spritz R.A., Weisman F., Weisman M., Slighton J.L., Blecht A.E., Smithie O., Baralle F.E., Shoulders C.C. & Proudfoot N.J. (1980) "The structure and evolution of the human beta-globin gene family" *Cell,* **21:** 653-668.

Fang K.S.Y., Vitale M., Fehlner P. & King T.P. (1988) "cDNA cloning and primary structure of a white-faced hornet venom allergen, antigen S" *Proc. Natl. Acad. Sci. USA,* **85:** 895-899.

Faulkner D.V. & Jurka J. (1988) "Multiple aligned sequence editor (MASE)" *Trends Biochem. Sci.,* **13:** 321-322.

Feng D.F., Johnson M.S. & Doolittle R.F. (1985) "Aligning amino acid sequences: Comparison of commonly used methods" *J. Mol. Evol.,* **21:** 112-125.

Finlay C., Hinds P.W., Levine A.J. (1989) "The p53 proto-oncogene can act as a suppressor of transformation" *Cell,* **57:** 1083-1093.

Frankel A.D. & Pabo C.O. (1988) "Fingering too many proteins" *Cell,* **53:** 675.

Freedman R.B. (1989) "Protein disulphide isomerase: Multiple roles in modification of nascent secretory proteins" *Cell,* **7:** 1069-1072.

Frisch L. *ed.* (1966) "The genetic code" Cold Spring Harbour Symposia on Quantitative Biology **31.**

Fryklund L. & Sievertson H. (1978) "Primary structure of somatomedin-B" *FEBS Lett.,* **87(1):** 55-60.

Garbedian M.J., Shirras A.D., Bownes M. & Wensink P.C. (1987) "The nucleotide sequence of the gene coding for Drosophila melanogaster yolk protein 3" *Gene,* **55:** 1-8.

George D.G., Barker W.C. & Hunt L.T. (1986) "The protein identification resource PIR" *Nucleic. Acids Res.,* **14(1):** 11-15.

Gilbert W. (1978) "Why genes in pieces?" *Nature,* **271:** 501.

Gordon C. *ed.* (1988) "Mapping and sequencing the human genome" Washington: National Academy Press.

Gribskov M., Devereux J. & Burgess R.R. (1984) "The codon preference plot; graphic analysis of protein coding sequences and prediction of gene expression" *Nucleic. Acids Res.,* **12:** 539-549.

Groneborn A.M. & Clore G.M. (1989) "Three dimensional structures of proteins in solution by nuclear magnetic resonance spectroscopy" *Protein Sequence Data Analysis,* **2:** 1-8.

Hammond S.M., Lambert P.A. & Rycroft A.N. "The cell envelope in bacterial disease" in "The bacterial cell surface" p.177 London: Croom Helm.

Hantgan R.R., Hammes G.G. & Scheraga H.A. (1974) "Pathways of folding of reduced bovine pancreatic ribonuclease" *Biochemistry,* **13:** 3421-3431.

Hein J. (1989) "A tree reconstruction method that is economical in the number of pairwise comparisons used" *Mol. Biol. Evol.,* **6(6):** 669-684.

Heinikoff S. & Wallace J.C. (1988) "Detection of protein similarities using nucleotide sequence databases" *Nucleic. Acids Res.,* **16(13):** 6191-6204.

Henneke C.M. (1989) "A multiple sequence alignment algorithm for homologous proteins using secondary structure information and optionally keying alignments to functionally important sites" *CABIOS,* **5(2):** 141-150.

Higgins D.G. & Sharp P.M. (1989) "Fast and sensitive multiple sequence alignment on a microcomputer" *CABIOS,* **5(2):** 151-153.

Hirschberg D.S. (1975) "A linear space algorithm for computing longest common subsequences" *Commun. A.C.M.,* **18:** 341-343.

Horvath B., Kondorosi E., John M., Schmidt J., Torok I., Gyorgypai Z., Barabas I., Wieneke U., Schell J. & Kondorosi A. (1986) "Organization, structure and symbiotic function of Rhizobium meliloti nodulation genes determining host specificity for alfalfa" *Cell,* **46:** 335-343.

Howard H.J. (1986) "Malaria: Antigens and host-parasite interactions" in "Parasite antigens" Pearson T.W. *ed.* pp144-149 New York: Dekker.

Jany K.D. (1984) "Complete nucleic acid sequence of glucose dehydrogenase from Bacillus megasterium" *FEBS Lett.,* **165**: 6-10.

Jefferson R., Goldsbrough A. & Bevan M. (1990) "Transcriptional regulation of a patatin-1 gene in potato" *Plant Mol. Biol.,* **14(6)**: 995-1006.

Jenne D. &Stanley K.K. (1987) "Human S-protein gene: Repeating peptide motifs in the 'pexin' family and a model for their evolution" *Biochemistry,* **26**: 6735-6742.

Jones R., Taylor W., Zhang X., Mesirov J. & Lander E. (1990) "Protein sequence comparison on the connection machine CM-2" in "Computers and DNA" Bell G. & Mar T.G. *eds.* pp99-102, Redwood City MA: Addison-Wesley.

Kabsch W. & Sander C. (1983) "How good are predictions of protein secondary structure?" *FEBS Lett.,* **155(2)**: 179-182.

Kabsch W. & Sander C. (1984) "On the use of sequence homologies to predict protein structure: Identical pentapeptides can have completely different conformations" *Proc. Natl. Acad. Sci. USA,* **81**: 1075-1078.

Kahn P. & Cameron G. (1990) "The EMBL data library" *Methods in Enzymol.,* **183**: 23-30.

Kaufmann S., Legrand M. & Fritig B. (1990) "Isolation and characterisation of six pathogenesis related (PR) proteins of samsun NN tobacco" *Plant Mol. Biol.,* **14(3)**: 381-390.

King T.P., Moran D., Wang D.F., Kochourmian L. & Chait L. (1990) "Structural studies of a hornet venom allergen antigen 5, dol mV and its sequence similarity with other proteins" *Prot. Seq. Data Analysis,* **3(3)**: 263-266.

Knuth D.E. (1973a) "Fundamental algorithms" pp.104-107, 2nd ed. Reading MA: Addison Wesley.

Knuth D.E. (1973b) "Sorting and searching" pp.406-411, 2nd ed. Reading MA: Addison Wesley.

Knuth D.E. (1973c) "Sorting and searching" pp.555-556, 2nd ed. Reading MA: Addison Wesley.

Kostertopfer M., Frommer W.B., Rochasosa M. & Willmitzer L. (1990) "Presence of a transposon-like element in the promoter region of an inactive patatin gene in Solanum tuberosum" *Plant Mol. Biol.,* **14(2)**: 239-247.

Kraut J. (1977) "Serine proteases: Structure and mechanism of catalysis" *Ann. Rev. Biochem.,* **46:** 331-358.

Kruskal J.B. (1983) "An overview of sequence comparison" in "Time warps string edits and macromolecules: The theory and practice of sequence comparison" Sankoff D. & Kruskal J.B *eds.* Ch.1 pp.1-45, Reading MA: Addison-Wesley.

Lewin B. (1990) "Genes" 4th *edn.,* Oxford: Oxford UP.

Lindquist S. & Craig E.A. (1988) "The heat shock proteins" *Ann. Rev. Genet.,* **22:** 631-637.

Lipman D.J., Kececioglu J.D. & Altschul S.F. (1989) "A tool for multiple alignment" *Proc. Natl. Acad. Sci. USA,* **86(12):** 4412-4415.

Long J. & Whitefield A. eds (1989) "Cognitive ergonomics and human computer interaction" Cambridge: Cambridge UP.

Lucas S., Camacho-Hennquez A., Lolspeich F., Henschen A. & Sanger H.L. (1985) "Amino acid sequence of the 'pathogenesis related' leaf protein p14 from viroid-infected tomato reveals a new type of structurally unfamiliar protein" *EMBO J.,* **4:** 2745-2749.

Lyall A., Hill C., Collins J.F. & Coulson A.F.W. (1986) "Implementation of an inexact string matching algorithm on the I.C.L. DAP" in "Parallel computing 85" pp.235-240, Amsterdam: Elsevier.

Lyall A. (1988) "Biological sequence comparison on a parallel computer" Doctoral Thesis, Edinburgh University.

Masek W.J. & Paterson M.S. (1983) "How to compute string edit distances quickly" in "Time warps string edits and macromolecules: The theory and practice of sequence comparison" Sankoff D. & Kruskal J.B *eds.* Ch. 14 pp.327-349, Reading MA: Addison-Wesley.

McQuay S.J. (1991) "Interpretation of alignments between sequences of unusual composition" *Biochem. Soc. Transactions,* **19(2):** 523-524.

Mount D.W. (1988) in "Computational molecular biology" Lesk A.M *ed.* Oxford: Oxford UP.

Murata M., Richardson J.S. & Sussman J.L. (1985) "Simultaneous comparison of three protein sequences" *Proc. Natl. Acad. Sci. USA,* **82:** 3073-3077.

Nakai K., Kidera A. & Kanehisa M. (1988) "Cluster analysis of amino acid indices for prediction of protein structure and function" *Prot. Engineering,* **2(2):**

93-100.

Needleman S.B. & Wunsch C.D. (1970) "A general method applicable to the search for similarities in amino acid sequences of two proteins" *J. Mol. Biol.,* **48:** 443-453.

Osborne M.R. (1985) "Finite algorithms in optimization and data analysis" pp.325-332, Wiley.

Pallabiraman N., Namboodiri K., Lowrey A. & Gaber B.P. (1990) "NRL_3D: A sequence structure database derived from the protein databank (PDB) and searchable within the PIR environment." *Prot. Seq. Data Analysis,* **3(5):** 387-405.

Patthy L. (1985) "Evolution of the proteases of blood coagulation and fibrinolysis by assembly from modules" *Cell,* **41:** 657-663.

Pearson W.R. (1990) "Rapid and sensitive sequence comparison with FastP and FastA" *Methods in Enzymol.,* **183:** 63-89.

Quispel A. (1988) "Bacteria-plant interactions in symbiotic nitrogen fixation" *Phys. Plantum,* **74:** 784-790.

Regier D.A., Akiyoshi D.E. & Gordon M.P. (1989) "Nucleotide sequence of the tsz gene from Agrobacterium rhizogenes (strain A4)" *Nucleic. Acids Res.,* **17(21):** 8885.

Reizer J., Saier M.H., Thompson J., Grenier F., Hengstenberg W. (1988) "The phosphoenolpyruvate - sugar phosphotransferase system in gram-positive bacteria. Properties, mechanism and regulation" *CRC Critical Reviews in Microbiology,* **15(4):** 297-338.

Revuz D. (1975) "Markov chains" Amsterdam: Elsevier.

Ripka W.C. (1986) "Computer-assisted model building" *Nature,* **321:** 93-94.

Roberts R. (1990) "Computational challenges of the human genome" presented at "Computing in Molecular Biology" Conference, Chester.

Saier M.H., Grenier F.C., Lee C.A. & Waygood E.P. (1985) "Evidence for the evolutionary relatedness of the proteins of the bacterial phosphoenol-pyruvate sugar phosphotransferase system" *J. Cell. Biochem.,* **27:** 43-49.

Sambrook J. and Gething M.J. (1989) "Chaperones, paperones" *Nature,* **342:** 224-225.

Sanger F., Nicklen S. & Coulson A.R. (1977) "DNA sequencing with chainterminating inhibitors" *Proc. Natl. Acad. Sci. USA,* **74(12):** 5463-5467.

Sanger F., Coulson A.R., Hong G.F., Hill D.F. & Peterson G.B. (1982) "Nucleotide sequence of bacteriophage lambda DNA" *J. Mol. Biol.,* **162:** 729-773.

Sankoff D. & Kruskal J.B. *eds.* (1983) "Time warps, string edits and macromolecules: The theory and practice of sequence comparison" Reading MA: Addison-Wesley.

Santibanez M. & Rhode K. (1987) "A multiple alignment program for protein sequences" *CABIOS,* **3(2):** 111-114.

Schulz G.E. & Schirmer R.H. (1979) "Noncovalent forces determining protein structure" in "Principles of protein structure" Ch.3 pp.27-53, 2nd ed. New York: Springer-Verlag.

Schwartz R.M. & Dayhoff M.O. (1978) "Matrices for detecting distant relationships" in "Atlas of protein sequence and structure" Vol.5 Supp.3 Ch.23 pp.353-358, National Biomedical Research Foundation.

Sedgewick R. (1983) "Dynamic programming" in "Algorithms" Ch.37 pp483-495, Reading MA: Addison-Wesley.

Shannon C.E. & Weaver S. (1949) "The mathematical theory of communication" p.9, Urbana: Urbana UP.

Smith R.F. & Smith T.F. (1990) "Automatic generation of primary sequence patterns from sets of related proteins" *Proc. Natl. Acad. Sci. USA,* **87(1):** 118-122.

Smith T.F., Waterman M.S. & Burks C. (1985) "The statistical distribution of nucleic acid similarities" *Nucleic. Acids Res.,* **13(2):** 645-656.

Sobel E & Martinez HM (1986) "A multiple sequence alignment program" *Nucleic. Acids Res.,* **14(1):** 363-374.

Stavich B.R., Hahn B.H., Shaw G.M., Mc Neely P.D., Modrow S., Wolf H., Parks E.S., Parks W.P., Josephs S.F., Gallo R.G. & Wong-Staal F. (1986) "Identification and characterisation of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS" *Cell,* **45:** 637-648.

Stockwell P.A. & Peterson G.B. (1987) "HOMED: A homologous sequence editor" *CABIOS,* **3:** 37-43.

Stryer L. (1981) "Biochemistry" 2nd ed. p.61 San Francisco: Freeman.

Subbiah S. & Harrison S.C. (1989) "A method for multiple sequence alignment with gaps" *J. Mol. Biol.,* **209:** 539-548.

Surin B.P. & Downie J.A. (1988) "Characterization of the Rhizobium leguminosarun genes nodLMN involved in efficient host-specific nodulation" *Mol. Microbiology,* **2(2):** 173-183.

Taylor W.R. (1986) "Identification of protein sequence homology by consensus template alignment" *J. Mol. Biol.,* **183:** 233-258.

Taylor W.R. (1987a) "Protein Structure prediction" in "Nucleic acid and protein sequence analysis: A practical approach" Bishop M.J & Rawlings C.J. *eds.* Ch.12 p.313, Oxford: IRL Press.

Taylor W.R. (1987b) "Multiple sequence alignment by a pairwise algorithm" *CABIOS,* **3(2):** 81-87.

Taylor S.S. Buechler J.A. & Yonemoto W. (1990) "cAMP dependent protein kinase: A framework for a diverse family of regulatory enzymes" *Ann. Rev. Biochem.,* **59:** 971-1006.

Thirup S. & Larsen N.E. (1990) "Alma: An editor for large sequence alignments" *Proteins Struc. Func. & Genet.,* **7(3):** 291-295.

Tullo A., Liuni S. & Attimonelli M. (1989) "Reorganization and merging of the EMBL and Genbank keyword indeces in a tree structure for more efficient retrieval of nucleic acid sequences" *Prot. Seq. Data Anal.,* **2(4):** 327-334.

Varenne S., Lloubes R. & Lazdunski C. (1984) "Translation is a non-uniform process" *J. Mol. Biol.,* **180:** 549-576.

Van-Driel R.A. & Goding J.W. (1987) "Plasma cell glycoprotein PC-1., primary structure deduced from cDNA clones." *J. Biol. Chem.,* **262:** 4882-4887.

Waterman M.S., Smith T.F. & Beyer W.A. (1976) "Some biological sequence metrics" *Adv. Math.,* **20:** 367-387.

Watson J.D. & Crick F.H.C. (1953) "Molecular structure of nucleic acid. A structure for deoxyribose nucleic acid" *Nature,* **171:** 737-738.

Welch T.A. (June 1984) "A technique for high-performance data compression" *Computer,* **17(6):** 8-19.

Wenzler H., Fisher L., Park W. & Mignery G. (1989) "Sucrose-regulated expression of a chimeric potato-tuber gene in leaves of transgenic tobacco plants" *Plant Mol. Biol.,* **13(4):** 347-354.

Wetlaufer D.E. (1973) "Nucleation, rapid folding and globular interchain regions in proteins" *Proc. Natl. Acad. Sci. USA,* **70:** 697-701.

Wilbur W.J. & Lipman D.J. (1983) "Rapid similarity searches of nucleic and protein data banks" *Proc. Natl. Acad. Sci. USA,* **80:** 726-730.

Wilbur W.J. (1985) "On the PAM matrix model of protein evolution" *Mol. Biol. Evol.,* **2(5):** 434-447.

Wyse (1984) "WY-75 Display terminal quick reference guide" *N.p.*: Wyse Technology CA 95134.

Young R.A. & Elliot T.J. (1989) "Stress proteins, infection and immune surveillance" *Cell,* **59:** 5-8.

Zhakut-Houri R., Orem M., Bienz B., Larie V., Hazum S. & Givol D. (1983) "A single gene and a pseudogene for the cellular tumour antigen p53" *Nature,* **306:** 594-597.

Zvelebil M.J., Barton G.J., Taylor W.R. & Sternberg M.J.E. (1987) "Prediction of protein secondary structure and active sites using the alignment of homologous sequences" *J. Mol. Biol.,* **195:** 957-961.