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COMPUTATIONAL ANALYSIS OF EXPRESSED SEQUENCE TAGS
FOR UNDERSTANDING GENE REGULATION

By

I. Elizabeth Cha

B.S., Chemistry, Chung Yuan Christian University, Taiwan, R.O.C., 1995

M.S., Computer Engineering and Computer Science, University of Louisville, 2002

A Dissertation
Submitted to the Faculty of the
Graduate School of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Department of Computer Engineering and Computer Science
University of Louisville
Louisville, Kentucky

August 2008

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A Dissertation Approved on

July 30, 2008

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Dr. Adel S. Elmaghraby, CECS Department

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DEDICATION

This dissertation is dedicated to my parents

Mr. Shihgene Cha

and

Mrs. Hsiujen Chen Cha

who have given me invaluable educational opportunities and unconditional love.

ACKNOWLEDGEMENTS

It is a pleasure to finish this dissertation because it is proof of the work that I have done these past few years during my doctoral training. For this I would like to thank my advisor Dr. Eric C. Rouchka. His knowledge in Bioinformatics is so comprehensive that he has answered many of my questions and corrected my misunderstanding in the field. His patience in research has served as a testimony for me on how to be a researcher in the future. Words of encouragement always came at the right time to push me to continue working on my research. He really knew the right timing to needle me about moving on in my research!!!

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actually applied for me to attend the graduate school at the University of Louisville. Without his push, I would not be here today.

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One last person I need to mention is Dr. Gwong-chien Sun. He was the one who suggested that I apply for the CSE Ph.D. program in the CECS department at the University of Louisville instead of going to another university when I could not find a job and struggled with whether to stay in the U.S. or go home after I received my master's degree. Regrettably, he was not able to see me finish my Ph.D. since he passed away in March, just over two months after I started the doctoral training in Spring 2003.

The research projects in current BLAST software comparison, computational predictions of alternative splicing patterns in rat PDE4 sequences and pseudogene detections and characterization were supported by the NIH-NCRR Grant P20RR16481 (Nigel G. F. Cooper, PI) and NIH-NIEHS Grant P30ES014443 (Kenneth S. Ramos, PI).

I will quote a few verses from the English Standard Version of the Bible as an acknowledgement to the One who has enabled me to stay in the Ph.D. program.

“... He will surely be gracious to you at the sound of your cry. As soon as He hears it, He answers you. And though the Lord give you the bread of adversity and the water of affliction, yet your Teacher will not hide Himself anymore, but your eyes shall

see your Teacher. And your ears shall hear a word behind you, saying “This is the way, walk in it,” when you turn to the right or when you turn to the left.” ~ Isaiah 30:19b~21

Today is July 4th, 2008 and I have finished writing the acknowledgement of my dissertation in Louisville, Kentucky, United States of America.

ABSTRACT

COMPUTATIONAL ANALYSIS OF EXPRESSED SEQUENCE TAGS FOR UNDERSTANDING GENE REGULATION

I. Elizabeth Cha

July 30, 2008

High-throughput sequencing has provided a myriad of genetic data for thousands of organisms. Computational analysis of one data type, expressed sequence tags (ESTs) yields insight into gene expression, alternative splicing, tissue specificity gene functionality and the detection and differentiation of pseudogenes. Two computational methods have been developed to analyze alternative splicing events and to detect and characterize pseudogenes using ESTs.

A case study of rat phosphodiesterase 4 (PDE4) genes yielded more than twenty-five previously unreported isoforms. These were experimentally verified through wet lab collaboration and found to be tissue specific. In addition, thirteen cytochrome-like gene and pseudogene sequences from the human genome were analyzed for pseudogene properties. Of the thirteen sequences, one was identified as the actual cytochrome gene, two were found to be non-cytochrome-related sequences, and eight were determined to be pseudogenes. The remaining two sequences were identified to be duplicates.

As a precursor to applying the two new methods, the efficiency of three BLAST algorithms (NCBI BLAST, WU BLAST and mpiBLAST) were examined for comparing

large numbers of short sequences (ESTs) to fewer large sequences (genomic regions). In general, WU BLAST was found to be the most efficient sequence comparison tool.

These approaches illustrate the power of ESTs in understanding gene expression. Efficient computational analysis of ESTs (such as the two tools described) will be vital to understanding the complexity of gene expression as more high-throughput EST data is made available via advances in molecular sequencing technologies, such as the current next-generation approaches.

TABLE OF CONTENTS

DEDICATION	iii
ACKNOWLEDGEMENTS	iv
ABSTRACT	ix
LIST OF TABLES	xvii
LIST OF FIGURES	xix
CHAPTER I INTRODUCTION	1
1.1 Basic Genetics	2
1.2 DNA	2
1.3 RNA	4
1.4 Amino Acids	5
1.5 Proteins	8
1.6 Central Dogma of Molecular Biology	8
1.7 DNA Sequencing	11
1.7.1 Evolution of Sequencing	12
1.7.2 Expressed Sequence Tags	15
1.7.3 Human Genome Project (HGP)	16
1.7.4 ENCODE Project	18
1.8 Genes	18
1.8.1 What is a Gene?	18
1.8.2 Alternative Splicing	21

1.8.3 Pseudogenes	21
1.9 Bioinformatics and Computational Biology	22
1.10 Introduction to Main Projects.....	24
1.10.1 Comparison of BLAST Software	24
1.10.2 Alternative Splicing Recognition	25
1.10.3 Pseudogene Detection and Characterization	26
1.11 Organization of Dissertation	26
CHAPTER II DATA SOURCES AND DATA PREPROCESSING	28
2.1 Sequence Data	28
2.1.1 dbEST	28
2.1.2 Reference Sequence (RefSeq)	30
2.1.3 UCSC Genome Browser.....	31
2.1.4 Data Information	32
2.2 Data Format.....	33
2.3 Preprocessing Data.....	36
2.3.1 Filtering by Organism.....	36
2.3.2 Masking Repetitive Elements.....	37
2.3.3 Filtering Low Complexity Regions	37
CHAPTER III COMPUTATIONAL SEQUENCE ALIGNMENT	39
3.1 Dynamic Programming Algorithm (DPA)	39
3.2 Heuristic Algorithm.....	41
3.2.1 FASTA	41
3.2.2 BLAST	42

3.2.3 Current BLAST Tools	43
3.2.4 mpiBLAST	43
3.3 EST to Genome Alignment Algorithms.....	44
3.3.1 est2genome	44
3.3.2 Spidey	45
3.3.3 sim4	45
3.3.4 GMAP.....	45
3.3.5 EXALIN	46
3.4 Case Study in Comparison of Current BLAST Software	46
3.5 Methods	47
3.5.1 Data.....	47
3.5.2 Types of Sequence Searches	50
3.5.3 System and Software	51
3.6 Results	52
3.6.1 EST vs. EST	52
3.6.2 Chromosome vs. Chromosome	58
3.6.3 Chromosome vs. EST	59
3.6.4 EST vs. Chromosome.....	61
3.6.5 mpiBLAST	63
3.7 Discussion	65
3.8 Conclusion.....	66
CHAPTER IV COMPUTATIONAL METHOD FOR ALTERNATIVE SPLICE	
PATTERN RECOGNITION	67

4.1 Introduction	67
4.1.1 History of Alternative Splicing	67
4.1.2 Splicing Mechanism	68
4.1.3 Alternative Splicing Modes	70
4.1.4 Characteristics of Alternative Splicing	72
4.1.5 Rates of Alternative Splicing	73
4.2 Research in Alternative Splicing Detection	73
4.2.1 Automatic Methods for Detecting Alternative Splicing	73
4.2.2 Research of Automatic Applications in Alternative Splicing Detection	74
4.3 Applications for Alternative Splicing	76
4.4 Case Study in Phosphodiesterase 4 (PDE4)	76
4.4.1 Phosphodiesterases (PDEs)	77
4.4.2 Motivation	78
4.4.3 Phosphodiesterase 4 (PDE4)	78
4.5 Methods for Computational Detection	80
4.5.1 Genomic Data	80
4.5.2 Splice Detection	82
4.6 Results in Exon Predictions	85
4.6.1 Spliced Pattern Observation	90
4.6.2 PDE4A	91
4.6.3 PDE4B	95
4.6.4 PDE4C	99
4.6.5 PDE4D	101

4.7 Wet Lab Methods	104
4.7.1 Polymerase Chain Reaction (PCR)	104
4.7.2 RT-PCR	105
4.7.3 DNA Sequencing.....	106
4.7.4 Splice Exon Verification	106
4.8 Results in Verified Predicted Exons.....	107
4.8.1 Tissue Types.....	107
4.8.2 Predicted Exons Verification.....	107
4.9 Discussion	114
CHAPTER V COMPUTATIONAL METHOD FOR PSEUDOGENE DETECTION	
AND CHARACTERIZATION	116
5.1 Pseudogenes	116
5.1.1 Processed Pseudogenes	117
5.1.2 Unprocessed Pseudogenes.....	118
5.1.3 Disabled Pseudogenes	119
5.1.4 Functional Pseudogenes	119
5.1.5 Motivations for Pseudogene Detection and Characterization	120
5.2 Current Automatic Pseudogene Detecting Methods	120
5.2.1 Harrison's Approach	120
5.2.2 Sakai's Approach.....	121
5.2.3 PPFINDER	122
5.2.4 PseudoPipe	123
5.2.5 Pseudogenes Analysis in ENCODE Project.....	124

5.3 Characteristics for Pseudogene Detection.....	125
5.3.1 Start Codon.....	126
5.3.2 Stop Codons.....	127
5.3.3 Poly-A Signals and Poly-A Tails	128
5.3.4 G+C Composition.....	130
5.3.5 Similar, Synonymous vs. Nonsynonymous Mutations.....	130
5.4 Sequence Data	135
5.5 Methodology	137
5.5.1 Data Preprocessing.....	137
5.5.2 ESTs to Genome Alignment.....	137
5.5.3 Clustering	145
5.5.4 Pseudogene Detection and Characterization	146
5.6 Results	147
5.7 Discussion	158
CHAPTER VI CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH ..	161
6.1 BLAST Comparison Project	161
6.2 Rat Phosphodiesterase 4 (PDE4) Project	162
6.3 Computational Method of Detecting Alternative Splicing Events.....	163
6.4 Computational Method for Pseudogene Detection and Characterization	163
REFERENCES	166
APPENDIX I ABBREVIATIONS	184
APPENDIX II GLOSSARY	186
CURRICULUM VITAE.....	201

LIST OF TABLES

Table 1.1: IUPAC-IUB Nucleic Acid and Amino Acid Codes	3
Table 1.2: The Genetic Code (RNA to Amino Acids).....	6
Table 1.3: Structure and Classification of the 20 Common Amino Acids	7
Table 2.1: Top 25 Organisms in dbEST (as of May 23 rd , 2008)	29
Table 2.2: Statistical Information of RefSeq Sequences (as of May 9 th , 2008).....	31
Table 2.3: Available Sequence and Annotation Downloads in UCSC Genome Browser	32
Table 3.1: Data Used for BLAST Comparison.....	48
Table 3.2: Fragment Information in mpiBLAST	49
Table 3.3 ncbiBLAST Performs Best when the Number of Sequences in the Database is Small (Measured in sec).....	53
Table 3.4: wuBLAST Performs Best when the Number of Sequences in the Databases is Greater (Measured in sec).....	54
Table 4.1: Rat PDE4 Family mRNA Sequence Information.....	81
Table 4.2: PDE4A Exon Locations.....	86
Table 4.3: PDE4B Exon Locations.....	87
Table 4.4: PDE4C Exon Locations.....	88
Table 4.5: PDE4D Exon Locations.....	89
Table 4.6: Single Exon Splicing Patterns and Tissue Types for PDE4A	92
Table 4.7: Multiple Exon Splicing Patterns and Tissue Types for PDE4A.....	93
Table 4.8: Single Exon Splicing Patterns and Tissue Types for PDE4B.....	96

Table 4.9: Multiple Exon Splicing Patterns and Tissue Types for PDE4B	97
Table 4.10: Splicing Patterns and Tissue Types for PDE4C	100
Table 4.11: Splicing Patterns and Tissue Types for PDE4D	102
Table 4.12: PCR Primer Pairs for Selected Predicted Exons.....	108
Table 4.13: Sequencing Results (Primer 14-1 in PDE4A)	111
Table 4.14: Sequencing Results (Primer 15-1 in PDE4B).....	112
Table 4.15: Sequencing Results (Primer 23-1 in PDE4B).....	113
Table 5.1: Start Codon Alternative Patterns and Their Scores	127
Table 5.2: Stop Codon Alternative Patterns and Their Scores	128
Table 5.3: Poly-A Signal Patterns.....	129
Table 5.4: The Genetic Code (RNA to Amino Acids).....	132
Table 5.5: Percentage of N and G+C Composition in Sequences	148
Table 5.6: Frequency of Start Codon Patterns	149
Table 5.7: Frequency of Stop Codon Patterns	150
Table 5.7: Frequency of Stop Codon Patterns (Continued).....	151
Table 5.8: Frequency of Poly-A Signal Patterns	152
Table 5.9: Poly-A Site Information for Each Sequence	153
Table 5.10: Counts of Similar Synonymous Mutations, Synonymous Mutations, Nonsynonymous Mutations, and Insertion for Each Sequence	155
Table 5.11: Score for Pseudogene Candidate Sequences	157

LIST OF FIGURES

Figure 1.1: A diagram of a sugar-ring molecule with carbons labeled numerically.....	4
Figure 1.2: Double strand DNA sequences.....	4
Figure 1.3: Central Dogma Of Molecular Biology.....	8
Figure 1.4: tRNA.	9
Figure 1.5: Sequence ladder by radioactive sequencing compared to fluorescent peaks.	12
Figure 1.6: An overview of how ESTs are constructed.	16
Figure 1.7: Two types of pseudogenes: processed pseudogenes and unprocessed pseudogenes.	22
Figure 2.1: Example GenBank file 1392161.	34
Figure 2.2: Example FASTA format 1392161.....	35
Figure 3.1: Cumulative results of wuBLAST and ncbiBLAST in EST vs. EST comparison.....	55
Figure 3.2: est166 target database vs. est136 and est147 query sequences.	57
Figure 3.3: est166 target database vs. est241 and est176 query sequences.	57
Figure 3.4: Cumulative results of wuBLAST and ncbiBLAST using chromosome vs. chromosome.....	59
Figure 3.5: chr22 target database vs. est136 and est147 query sequences.	60
Figure 3.6: chr1 target database vs. est136 and est147 query sequences.	60
Figure 3.7: Cumulative results of wuBLAST and ncbiBLAST for EST vs. chromosome.	62

Figure 3.8: est166 target database vs. est136 query sequence in mpiBLAST.....	64
Figure 3.9: chr1 target database vs. est136 query sequence in mpiBLAST.	64
Figure 4.1: Central Dogma.	67
Figure 4.2: Canonical spliceosome for intron splicing.	69
Figure 4.3: Alternative splicing events.	70
Figure 4.4: General scheme of cyclic 3', 5' nucleotide metabolism.....	77
Figure 4.5: PDE4 isoform diversity: long, short, super-short and dead-short.	79
Figure 4.6: Process flow chart in computational detecting section.	83
Figure 4.7: Tissue distribution of ESTs for each of the four PDE4 families.....	90
Figure 4.8: PDE4A isoforms, EST patterns and sequenced regions.....	94
Figure 4.9: PDE4B isoforms, multiple EST patterns and sequence hits.	98
Figure 4.10: PDE4C isoforms, multiple EST patterns and sequence hits.	100
Figure 4.11: PDE4D isoforms, multiple EST patterns and sequence hits (chromosome 2).	103
Figure 4.12: PDE4D isoforms, multiple EST patterns and sequence hits (chromosome 2 RANDOM).	104
Figure 4.13: Basic steps in the PCR cycle.	105
Figure 4.14: RT-PCR for PDE4A pattern 14.....	109
Figure 4.15: RT-PCR for PDE4B patterns 15 and 23.....	109
Figure 4.16: RT-PCR for PDE4D patterns 21 and 22.	110
Figure 5.1: Central Dogma.	117
Figure 5.2: Pseudogene formation.	117
Figure 5.3: Flow chart of Sakai <i>et al.</i> method for processed pseudogene detection.....	122

Figure 5.4: Mutations occurring in two homologous sequences.	131
Figure 5.5: Comparison between ancestral sequence and descendant sequence (1).	132
Figure 5.6: Comparison between ancestral sequence and descendant sequence (2).	133
Figure 5.7: Similar synonymous mutation vs. nonsynonymous mutation.....	134
Figure 5.8: Process for pseudogene detection.....	136
Figure 5.9: Sample <code>blastn</code> result for GI 5555714.....	138
Figure 5.10: Flow chart for filtering <code>blastn</code> results.	140
Figure 5.11: Sample <code>blastn</code> result GI no. 18288550.	142
Figure 5.12: Sample EXALIN result for GI 18288550.	142
Figure 5.13: Sample EXALIN result for GI 18288550.	143
Figure 5.14: Flow chart for removing EXALIN redundant records.	144
Figure 5.15: Clustering by DNA walking.....	146

CHAPTER I

INTRODUCTION

Since the laws of inheritance were described by the Augustinian monk Gregor Mendel in 1866 (1-4), the genomic basis for heredity has been a topic of interest. The discovery of nucleic acids by Friedrich Miescher (5) in 1869 moved the study of genetics to a molecular level. Fueled by the basic ‘truth’ of the Central Dogma of Molecular Biology (6), molecular biologists have worked towards understanding the complex relationship between deoxyribonucleic acids (DNA), ribonucleic acids (RNA) and amino acids as they create proteins providing structure, function and regulation to a biological system.

The possibility of decoding the genomic DNA for a species through DNA sequencing has led to the ability to glimpse at the genetic blueprint for an individual. The vast amount of genomic data resulting from projects such as the human genome project provides a treasure chest of data waiting to be explored. This has led to the emergence of the field of Bioinformatics to provide computational means for handling and mining such data. The focus of this dissertation is to provide Bioinformatics' approach to understanding the complexity involved in gene regulation. In order to adequately address the problem at hand, the rest of this chapter is devoted to a background on basic genetics, molecular biology, DNA sequencing, genes, and bioinformatics, as well as a brief introduction into three interwoven projects dealing with gene regulation.

1.1 Basic Genetics

The field of genetics traces its roots to 1866 with the published observations of the Augustinian monk Gregor Mendel (1-4). Mendel performed pea plant experiments to study the inheritance of genetic traits, such as tall vs. dwarf plants, wrinkled vs. round peas, violet vs. white flowers, etc. He concluded that the characteristics of living organisms are passed from generation to generation through replication.

Several major discoveries since have advanced the study of genetics at a molecular level. In 1869, nucleic acids were isolated and studied by Friedrich Miescher (5). In 1944, Avery, MacLeod and McCarty determined that DNA is the carrier of hereditary information (7;8) through four nucleic acid bases: adenine (A), cytosine (C), guanine (G) and thymine (T). This tetranucleotide hypothesis was verified in the late 1940s and early 1950s by Erwin Chargaff and his colleagues (9;10). In 1953, Watson and Crick discovered the structure of a double helix for DNA (11). These discoveries opened the door to many more advances in genetics over the past 60 years in the field of molecular biology, the branch of life science which studies cells at a molecular level, including nucleic acids, amino acids, proteins and fundamental biochemical reactions within a cell.

1.2 DNA

Deoxyribonucleic acid, or DNA, is composed of four nucleic acid bases – adenine (A), cytosine (C), guanine (G) and thymine (T) – which are arranged in different ways to form sequences. The International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) published a code in 1985 for

representing DNA sequences (12) (Table 1.1). A single letter signifies a single nucleotide or nucleotide alternative, as shown in columns one and two.

Table 1.1: IUPAC-IUB Nucleic Acid and Amino Acid Codes

Nucleotide Acid Codes		Amino Acid Codes		
Code	Base	Full name	3-letter abbreviation	1-letter abbreviation
A	Adenine	Glycine	GLY	G
C	Cytosine	Alanine	ALA	A
G	Guanine	Valine	VAL	V
T	Thymine in DNA; Uracil in RNA	Leucine	LEU	L
M	A or C	Isoleucine	ILE	I
R	A or G	Phenylalanine	PHE	F
W	A or T	Proline	PRO	P
S	C or G	Serine	SER	S
Y	C or T	Threonine	THR	T
K	G or T	Cysteine	CYS	C
V	A or C or G; not T	Methionine	MET	M
H	A or C or T; not G	Tryptophan	TRP	W
D	A or G or T; not C	Tyrosine	TYR	Y
B	C or G or T; not A	Asparagine	ASN	N
N	A or C or G or T	Glutamine	GLN	Q
		Aspartic acid	ASP	D
		Glutamic acid	GLU	E
		Lysine	LYS	K
		Arginine	ARG	R
		Histidine	HIS	H

As Watson and Crick revealed (11), DNA is structured as a double helix, where two strands are held together and rotated around a horizontal axis. These two strands are “anti-parallel,” with one strand running from a 5’ to 3’ direction and the other strand

running from 3' to 5', where 3' and 5' represent structural properties in nucleic acids¹. The two strands are held together by the energy of hydrogen bonds in complementary sequences where an A always pairs with a T, and a C always pairs with a G (and vice-versa, see Figure 1.2).

Figure 1.1: A diagram of a sugar-ring molecule with carbons labeled numerically.

Figure 1.2: Double strand DNA sequences.

1.3 RNA

RNA stands for ribonucleic acid. Three of the four DNA bases are the same in RNA, with the exception that thymine (T) is substituted by uracil (U). Unlike DNA, RNA can leave the nucleus of a cell to perform functions and is normally a single-stranded molecule. There are three major, functionally-distinct varieties of RNA: messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA). mRNA is involved in the

¹ Directionality in molecular biology refers to the end-to-end chemical orientation of a single strand of nucleic acid. The chemical convention of naming carbon atoms in the nucleotide sugar-ring numerically gives rise to a 5' end and a 3' end (usually pronounce “five prime end” and “three prime end”). Therefore, the 5' end designates the end of the DNA or RNA strand that has the fifth carbon in the sugar-ring of the (deoxy)ribose at its terminus. The 3' end has the third carbon in the sugar-ring as its terminus (Figure 1.1).

transmission of DNA information. rRNA functions in the synthesis process of producing proteins. tRNA enables another part of protein synthesis by bringing together chains of amino acids (13). There are some other types of RNAs, such as RNA interference (RNAi), microRNA (miRNA) and silencing RNA (siRNA). They will be discussed in Section 1.6, Central Dogma.

1.4 Amino Acids

Amino acids are the basic elements of proteins. They form short polymer chains called peptides or longer sequences called polypeptides. In an mRNA sequence, every three nucleic acids form a codon² that describes one amino acid. Since there are four nucleic acids, A, C, G and T, a codon can have up to $4 \times 4 \times 4$ or 64 different combinations, yet there are only twenty common amino acids. In 1962, Marshall Nirenberg (14-16) uncovered the genetic code, which allows for multiple codons to code for the same amino acid as shown in Table 1.2. During the translation process, a sequence begins with the initiator codon AUG and ends with any of the three terminator codons: UAA, UAG or UGA.

² A codon is a sequence of three adjacent nucleotides constituting the genetic code that determines the insertion of a specific amino acid in a polypeptide chain during protein synthesis or gives the signal to start or stop protein synthesis.

Table 1.2: The Genetic Code (RNA to Amino Acids)

		Second letter				
		U	C	A	G	
First letter	U	UUU	UCU	UAU	UGU	U
		UUC	UCC	UAC	UGC	C
		UUA	UCA	UAA	UGA	A
		UUG	UCG	UAG	UGG	G
	C	CUU	CCU	CAU	CGU	U
		CUC	CCC	CAC	CGC	C
		CUA	CCA	CAA	CGA	A
		CUG	CCG	CAG	CGG	G
	A	AUU	ACU	AAU	AGU	U
		AUC	ACC	AAC	AGC	C
		AUA	ACA	AAA	AGA	A
		AUG	ACG	AAG	AGG	G
	G	GUU	GCU	GAU	GGU	U
		GUC	GCC	GAC	GGC	C
		GUA	GCA	GAA	GGA	A
		GUG	GCG	GAG	GGG	G

...CH-CO-NH... is the common chemical composition of all twenty amino acids, where C stands for carbon, H for hydrogen, O for oxygen and N for nitrogen. Attached to each CH is attached a small group of atoms – often called R by chemists, where R is a side-chain in the molecule. The side-chains provide for differentiation for the common twenty amino acids are listed in Table 1.3.

Table 1.3: Structure and Classification of the 20 Common Amino Acids

One Letter	Three Letters	Name	Linear Structure
A	Ala	Alanine	CH ₃ -CH(NH ₂)-COOH
C	Cys	Cysteine	HS-CH ₂ -CH(NH ₂)-COOH
D	Asp	Aspartic acid	HOOC-CH ₂ -CH(NH ₂)-COOH
E	Glu	Glutamic acid	HOOC-(CH ₂) ₂ -CH(NH ₂)-COOH
F	Phe	Phenylalanine	Ph-CH ₂ -CH(NH ₂)-COOH
G	Gly	Glycine	NH ₂ -CH ₂ -COOH
H	His	Histidine	NH-CH=N-CH=C-CH ₂ -CH(NH ₂)-COOH
I	Ile	Isoleucine	CH ₃ -CH ₂ -CH(CH ₃)-CH(NH ₂)-COOH
K	Lys	Lysine	H ₂ N-(CH ₂) ₄ -CH(NH ₂)-COOH
L	Leu	Leucine	(CH ₃) ₂ -CH-CH ₂ -CH(NH ₂)-COOH
M	Met	Methionine	CH ₃ -S-(CH ₂) ₂ -CH(NH ₂)-COOH
N	Asn	Asparagine	H ₂ N-CO-CH ₂ -CH(NH ₂)-COOH
P	Pro	Proline	NH-(CH ₂) ₃ -CH-COOH
Q	Gln	Glutamine	H ₂ N-CO-(CH ₂) ₂ -CH(NH ₂)-COOH
R	Arg	Arginine	HN=C(NH ₂)-NH-(CH ₂) ₃ -CH(NH ₂)-COOH
S	Ser	Serine	HO-CH ₂ -CH(NH ₂)-COOH
T	Thr	Threonine	CH ₃ -CH(OH)-CH(NH ₂)-COOH
V	Val	Valine	(CH ₃) ₂ -CH-CH(NH ₂)-COOH
W	Trp	Tryptophan	Ph-NH-CH=C-CH ₂ -CH(NH ₂)-COOH
Y	Tyr	Tyrosine	HO-Ph-CH ₂ -CH(NH ₂)-COOH

Abbreviations: C, carbon; H, hydrogen; O, oxygen; N, nitrogen; S, sulfur;
Ph, phenyl group or phenyl ring

1.5 Proteins

Proteins are linear polymers built from amino acids. They carry out all of a cell's activities. Proteins serve different roles in a cell. As enzymes, proteins can accelerate the rate of metabolic reactions. As hormones, proteins perform regulatory functions inside a cell, such as activating genes. As membrane receptors and transporters, proteins determine what types of substances enter or leave the cell and give instructions on how a cell should react. There are many additional functions provided by proteins (17).

The specific amino acid sequence forming a protein gives the cell its unique property. Proteins may work alone in a cell or they can also work together to achieve a particular function. Proteins will often associate to form stable complexes in the cell.

1.6 Central Dogma of Molecular Biology

The journey from genes to proteins involves several steps in the Central Dogma of Molecular Biology. In transcription, a DNA gene is copied to a primary RNA transcript. During the splicing process, exons (coding regions) join together and introns (non-coding regions) are discarded, turning the primary RNA transcript into messenger RNA (mRNA). Later, through the process of translation, mRNA is transformed into a protein sequence. This Central Dogma was first described by Francis Crick in 1958 (6).

Figure 1.3: Central Dogma Of Molecular Biology.

RNA is synthesized in the nucleus of cells. When a promoter³ is recognized, RNA polymerase⁴ binds to it, catalyzes the insertion to open the DNA double helix strands, and starts to make an RNA transcript. The process will not stop until the enzyme traverses the entire gene and meets a termination signal. Then the transcribed RNA molecule is released from the DNA.

The initial RNA transcript passes through an excision and rejoining process, called splicing. The introns are removed and the exons are recombined together, creating mRNA. This mRNA associates with the ribosome and presents a series of codons that will only match to specific amino acids. Each amino acid is attached to a tRNA molecule. The tRNA with three consecutive ribonucleotides that complement the codon will be paired with the section of mRNA (See Figure 1.4). The amino acids combine to form protein (18;19).

Figure 1.4: tRNA.

³ A promoter is a sequence of DNA that specifies the sites of transcription initiation for an RNA polymerase.

⁴ A polymerase is an enzyme that can synthesize new DNA strands using a DNA template while several such enzymes exist.

The concept of the Central Dogma has been challenged by a series of discoveries revealing the complexity of gene expression. The first challenge came in 1970 with the discovery of reverse transcription. Reverse transcription allows a double stranded DNA molecule to be created from a single strand RNA template. At first, reverse transcription sequences were found mostly in viruses. But after large-scale sequencing techniques were established, close to half of the sequences in the human genome were found to be retroelements and their remnants (20).

In 1988, the discovery of RNA interference (RNAi) by Andrew Fire and Craig Mello solved the problem of retroelement recognition (21-26). They found that it is common for animals and plants to utilize enzymatic machinery in order to silence translation. Short RNAs (22-25 base pairs in length), called small interfering RNA (siRNA), can traverse intracellular spaces to enter cells and trigger degradation of homologous RNAs before they are translated to proteins. Both RNAi and siRNA are microRNA (miRNA).

In addition, prions, the microscopic protein particles similar to viruses but lacking nucleic acids, may be the simplest exception to the Central Dogma. Prions have been thought to be the infectious agent responsible for scrapie and certain other degenerative diseases of the nervous system, such as Creutzfeldt-Jakob and mad cow disease.

The mystery of non-coding RNA is still a hot topic. RNAi is the most recent RNA-based phenomenon to catch biologists' attention. There may be more secrets unveiled to challenge the traditional view of a Central Dogma in the near future (20).

1.7 DNA Sequencing

DNA sequencing is the method used to determine the order of the nucleotide bases, A, C, G and T, in a DNA oligonucleotide⁵. The methods were first developed in 1977 with the publication of two approaches. One was developed by Frederick Sanger (27) which became known as Sanger sequencing. It is based on enzymatic synthesis from a single-strand DNA template with chain termination using four dideoxynucleotides (ddNTPs)⁶ as chain terminators – terminator A, terminator C, terminator G, and terminator T. Another method was designed by Maxam and Gilbert (28) that involved chemical degradation of end-radio-labeled DNA fragments. Both methods depended on four-lane, high-resolution polyacrylamide gel electrophoresis⁷ to separate the labeled fragments so the sequence can be read in a staggered ladder-like platform. The Sanger method allows for the DNA to be combined in a single experiment due to the unique dyes identifying each nucleotide. Therefore, it became the major DNA sequencing method (32).

In Sanger sequencing, the ddNTP lacks a 3'OH group. Thus, DNA polymerases cannot extend the template copy chain beyond the incorporated ddNTP. In other words, ddATP terminates chains at every A (adenine) occurrence in the sequences. The other three ddNTPs, ddCTP, ddGTP, and ddTTP, work in the same way for their respective bases. They are placed in a test tube with a DNA fragment and DNA polymerase. The strands then placed on a gel to run electrophoresis. Since the ddNTP is phosphorescently

⁵ An oligonucleotide (or oligo) is a short polymer segment of RNA or DNA, length ranges from two to twenty bases.

⁶ ddNTP, which stands for dideoxynucleotide, represents one of the four portions: ddATP, ddCTP, ddGTP and ddTTP.

⁷ Electrophoresis is a method of separating substances, especially proteins, and analyzing molecular structure based on the rate of movement of each component in a colloidal suspension while under the influence of an electric field. This method was introduced by Arne Tiselius in 1937 for separating proteins in solution (29-31).

labeled, the locations where the chain terminators were incorporated for one of the four particular nucleotide, is known and the DNA sequence can be determined based on the order of incorporated ddNTPs (32).

Figure 1.5: Sequence ladder by radioactive sequencing compared to fluorescent peaks.

1.7.1 Evolution of Sequencing

The demand for DNA sequence information has never been greater, especially with the popularity of the Human Genome Project (Section 1.7.3). However, the Sanger sequencing method is costly, time consuming and labor intensive. Therefore, several emerging technologies have tried to improve Sanger's method to deliver fast and affordable genome sequencing (33). These methods include traditional electrophoretic approaches (slab gel electrophoresis, capillary electrophoresis, and microfabricated capillary arrays) and nonelectrophoretic methods (pyrosequencing, massively parallel signature sequencing, and sequencing by MLDI-TOF mass spectrometry) (34).

Acrylamide slab gel electrophoresis uses radioactive (^{32}P) labels and autoradiograms for the *de novo* sequencing of DNA fragments. The fluorescently labeled DNA fragments are loaded into acrylamide gel. An electric current causes the fragments migrating through the gel to separate by the weight of the nucleic acids. Then a detection system scans these radioactive labeled bands and reconstructs the original nucleotide sequences. This method can process up to 96 samples in a 9- to 11-hour run and can read sequences ranging in length from 650 to 750 bases (34).

The capillary array electrophoresis (CAE) method applies the same process as slab gels, except CAE adds a single sample in each capillary to avoid tracking problems. In addition, CAE allows more rapid heat dissipation. Therefore, the CAE method accommodates higher operating voltages and produces a faster run time. The CAE method can run 128 capillaries at one time and provide readings 500 bases long (34).

Microfabricated capillary arrays tried to perform the sample preparation, thermal cycling and electrophoretic separation on a single chip. This method etches a very large number of capillaries onto a single wafer at a low cost for parallel operation. In addition, this method allows the instrument designer to control the size of the injected DNA plug. It can handle 16-channel devices at the same time and produce average read lengths over 450 bases (34).

In the 1980s and 1990s researchers started to work on finding nonelectrophoretic separation methods for sequencing. Pyrosequencing monitors the concentration of pyrophosphate (PPi) during real-time DNA synthesis. First, it anneals an oligonucleotide primer to a single-stranded DNA template in the presence of DNA polymerase. Then, the first of four deoxynucleotide triphosphates (for example, dATP) is added to the reaction

mixture. When dATP encounters the right complement in the target sequence (adenine), the DNA polymerase releases PPi because the nucleotide reacts to the 3' end of the nascent strand. The concentration of PPi in the system is monitored using enzymatic reactions. Next, dATP is removed and another deoxynucleotide triphosphate is added to repeat the process. Each of the other three deoxynucleotide triphosphates (dCTP, dGTP and dTTP) will release PPi in the presence of their respective nucleic acids (34).

Massively parallel signature sequencing (MPSS) is another sequencing strategy. This method examines cDNA libraries by combining the highly parallel nature of expression microarrays with the robust statistical digital output from gel-based sequencing. The process uses 32-mer tags to compose of strings of eight 4-mer “words”. Each bead carries approximately 100,000 cDNA copies of a unique mRNA. These microbeads are sorted by a fluorescence-activated cell sorter. Then they are delivered to a micromachined flow cell to form a planar. Close to one million cDNA-coated beads can be analyzed in a single flow cell using conventional fluorescence microscopy (34).

Another method for sequencing DNA is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). It extends concepts from mass spectrometry used to detect macromolecules. The DNA sample is treated with a short pulse of ultra-violet (UV) laser that is absorbed by the 3-hydroxypicolinic acid to form ionized DNA fragments. These ions are extracted by a high-voltage pulse and accelerated in an electric field. A mass spectrum is generated and peaks identifying the weights for the ionized DNA fragments are evaluated by comparing them to the pre-built internal standards (34).

One idea for the next-generation of sequencing is to create a factory-like sequencing center. This would involve automated capillary electrophoresis and analyzing the sequences in parallel (35).

Next-generation sequencing may also improve the length of reads. Generally speaking, sequencing-by-synthesis technology provides reads 400 bases long. Advances in combining the emulsion polymerase chain reaction (PCR) with pyrosequencing for the bias-free amplification of single DNA molecules will eliminate the competition of multiple templates with limited DNA polymerases. This process could be performed in parallel by generating a visual signal for processing by a computer system (35).

These improvements could reduce cost and allow assessment at functional levels. As a result, they would facilitate individual genome sequencing for personalized treatment (34-36).

1.7.2 Expressed Sequence Tags

The mRNA molecule is not stable outside of the cell. In order to preserve genetic information, mRNA can be reverse transcribed into complementary DNA (cDNA). The cDNA usually contains an oligo(dT) primer⁸. This end of the cDNA derives from the polyadenyl (poly-A) tail⁹ at the end of the mRNA. The resulting cDNA is cloned into a vector. Expressed sequence tags (ESTs) are segments of cDNA that have been sequenced. Sequence of cDNA can be identified from either the beginning portion (which is called a 5' EST) or the end portion (which is called a 3' EST) (18;19;37-39).

⁸ A primer is a segment of DNA or RNA that is complementary to a given DNA sequence and that is needed to initiate replication by DNA polymerase.

⁹ A polyadenyl (poly-A) tail is a sequence of 50 to 250 adenine (A) residues that are added to the 3' end of an mRNA sequence after being cleaved. This reaction is catalyzed by polyadenylate polymerase.

An EST, which is usually 300 to 600 nucleotides long, represents a segment of a cDNA from a corresponding mRNA. It helps to identify unknown genes expressed in a given cell and can be used to map their positions within a genome.

Figure 1.6: An overview of how ESTs are constructed.

1.7.3 Human Genome Project (HGP)

The push to map the human genome was initiated by a 1988 report from the National Academy of Science (NAS) Committee which maintained that the human genome could be sequenced in 15 years, plus an additional two years to fill in gaps and correct mistakes. The project was submitted to Congress where it was approved and received \$3 billion in funding. It began in 1990 (40).

The goals of the HGP were to “*identify* all the approximately 20,000-25,000 genes in human DNA, *determine* the sequences of the 3 billion chemical base pairs that make

up human DNA, *store* this information in databases, *improve* tools for data analysis, *transfer* related technologies to the private sector, and *address* the ethical, legal, and social issues (ELSI) that may arise from the project” (40;41).

In February 2001, the work of the HGP and Celera published the first draft of the human genome (41-44). In 2003, 95% of the gene-containing part of the human sequence was mapped to 99.99% accuracy.

After the human genome was available, researchers started to think about what could be done with this information. One option is to design personalized medicine for more effective treatment. In order to design an individualized medicine, however, researchers must be able to sequence an individual genome. On June 1st, 2007, 454 Life Science Corporation in collaboration with scientists at the Human Genome Sequencing Center at Baylor College of Medicine (45), announced that they had sequenced James Watson’s personal genome. Later at the end of the same month, the Cold Spring Harbor Laboratory (CSHL) announced that an online sequence browser for Watson’s genome was available (46). Watson, who was the CSHL Chancellor and is currently serves as an advisor for the newly-formed Allen Institute for Brain Science¹⁰, said, “I am putting my genome sequence on line to encourage the development of an era of personalized medicine, in which information contained in our genomes can be used to identify and prevent disease and to create individualized medical therapies” (45;46).

¹⁰ Allen Institute for Brain Science, located in Seattle, Washington, was founded in 2003 by philanthropists Paul G. Allen and Jody Allen Patton as a nonprofit corporation (501(c) (3)) and medical research organization. (http://www.alleninstitute.org/content/about_the_institute.htm)

1.7.4 ENCODE Project

The ENCyclopedia of DNA Elements (ENCODE) project was initiated in September 2003. It brought several dozen laboratories together to identify the functional elements in the human genome. Two components – a pilot phase and a technology development phase – were proposed when the project started.

The aim of the pilot phase was to evaluate a variety of different methods for use in later stages. A portion of the genome equal to about 1% (30MB) was carefully chosen and analyzed using new and existing methods. Transcripts and gene models, protein-binding sites, epigenetic marks, promoters and enhancers, and DNA replication regions were the fundamental items involved in the research. The conclusions of this pilot project were published in June 2007 (47-56).

The technology development phase promoted several new technologies and protocols to generate high throughput data on functional elements for use in the ENCODE project. A third phase the production phase began in September 2007. A Data Coordination Center at the University of California at Santa Cruz (UCSC) was created to track, store and display the ENCODE data. In addition, a Data Analysis Center was established. All data generated by the ENCODE participants is released by the Data Coordination Center (49).

1.8 Genes

1.8.1 What is a Gene?

Genes are pieces of DNA that provide the fundamental physical and functional information of heredity. Through the processes of transcription and translation, the DNA

of a gene controls the manufacturing of specific proteins for regular usage throughout all cells and organ systems. Each sequence, appearing in a particular location of a particular chromosome, encodes a unique function.

Genes include coding regions (exons) and non-coding regions (introns). Coding regions contain information used to specify amino acids in the synthesis of proteins. Non-coding regions do not. Genes are spliced to have alternative combinations of exons to produce various proteins in specific organs (57;58). This allows cells in different organs to serve different functions.

In 2007, Gerstein *et al.*(51) published a review of the changing definition of genes from the 1860s to the early 2000s, just before the ENCODE project (Section 1.3.4). Between the 1860s-1900s, each gene was treated as a discrete unit of heredity, based on the concept developed by Gregor Mendel in 1866 (1-4). The word ‘gene’ was introduced by Wilhelm Johannsen in 1909, by combining two Greek words, *genesis* (meaning ‘birth’) and *geno* (meaning ‘origin’). Mendel’s work was rediscovered by Correns, von Tschermak-Seysenegg and de Vries in 1900 (59-61).

In the 1910s, the gene became defined as a distinct locus¹¹. Thomas Hunt Morgan demonstrated that genes are carried on chromosomes and are the mechanical basis of heredity. Hunt and his students (62) represented genes using a linear model from studying mutation in fruit flies (*Drosophila melanogaster*). The ability of genes to cross-over to each other was proportional to the distance that separated them. Mutations and recombination were two methodologies that early geneticists proposed, “so the gene was essentially a locus whose size was determined by’ mutations that inactivated a trait of interest and by the size of the recombining regions.”

¹¹ A locus is a position that a given gene occupies on a chromosome.

In the 1940s, the gene was seen as a blueprint for a protein. This gave rise to the statement “one gene, one enzyme” and soon evolved to “one gene, one polypeptide.” Hence, in the biochemical pathway, genes provided the information behind the individual molecules.

In the 1950s, after Muller published an observation of mutations caused by X-rays, the gene was described as a physical molecule. Later, in the mid 1950s, Hershey and Chase found from their study of bacteria that inheritance came from DNA and not protein. After Watson and Crick announced the three-dimensional structure of DNA, the gene was explained as transcribed code in the 1960s. Crick further presented a hypothesis of gene expression from nucleic acid to protein, which soon became known as the “Central Dogma” (Section 1.6). Molecular biology developed rapidly during this period of time.

Between the 1970s-1980s, Sanger developed sequencing and cloning technology (63). In parallel, computational tools were developed to utilize algorithms in order to identify genes based on sequential characteristics. Therefore, the gene became defined as an open reading frame (ORF) sequence pattern.

After the HGP was announced, a gene became further defined as “a DNA segment that contributes to phenotype/function. In the absence of demonstrated function a gene may be characterized by sequence, transcription or homology” according to the Human Genome Nomenclature Organization (64).

After the ENCODE pilot project (Section 1.7.4), the definition suggested by the ENCODE consortium was concisely summarized as “the gene is a union of genomic sequences encoding a coherent set of potentially overlapping functional products” (51).

1.8.2 Alternative Splicing

Alternative splicing is process of mRNA transcription through which the coding regions for a eukaryotic gene, called exons, are extracted and connected to produce alternative mRNA molecules. The event leads to a number of distinguishable proteins for a single transcriptional region. The resulting mRNA molecules, known as isoforms or splice variants, are becoming increasingly valued as an important biological method of producing complexity and diversity.

There are different kinds of alternative splicing: alternative promoter selection, alternative polyadenylation sites, exon skipping, exon retention, exon extension, exon truncation and intron retention. Alternative splicing is discussed in greater detail in Chapter 4.

1.8.3 Pseudogenes

A pseudogene is an inactive sequence of genomic DNA that shares sequence homology to a functional gene. In 1985, Vanin suggested “that pseudogenes (should) be used only to describe sequences found to be both related and defective” (65). Because of the high percentage of similarity to an existing decoded gene, pseudogenes are considered as evolutionary relatives to normally functioning genes.

Pseudogenes are created in two fundamentally different ways: retrotransposition and duplication of genomic DNA. Pseudogenes resulting from retrotransposition are called processed pseudogenes and pseudogenes resulting from DNA duplication are known as duplicated pseudogenes. Figure 1.7 provides a basic illustration of how

processed and unprocessed pseudogenes are produced. Pseudogene detection and characterization are research interests of this dissertation discussed in Chapter 5.

Figure 1.7: Two types of pseudogenes: processed pseudogenes and unprocessed pseudogenes.

1.9 Bioinformatics and Computational Biology

Bioinformatics is a compound word combining “bio” and “informatics.” As the word suggests, this field of research is related to both biology and information. A related term, “Computational Biology,” invokes the idea of using computers to perform calculations for biology.

From the perspective of computer science, biological data can be formalized into an alphabetic containing letters, such as A, C, G and T for DNA; A, C, G and U for RNA; or 20 letters for amino acids. This alphabet can then be used to produce “words” for sequences of DNA, RNA, or amino acids (66).

Bioinformatics and Computational Biology refer to applications of computers and computational techniques to deal with the storage and analysis of biological data (57). They use tools to implement algorithms into software to retrieve and analyze the

biological data and manage data storage. Compared to other sciences, Bioinformatics and Computational Biology are relatively young disciplines.

In earlier years, “Bioinformatics” was used more frequently in Europe and the United Kingdom. Research in Bioinformatics centered on data management. Computational Biology, on the other hand, which was used more often in the United States, referred mostly to algorithm development. In recent years, the boundary between Bioinformatics and Computational Biology has become less clearly defined. Both fields now work on managing data and constructing algorithms to analyze it. Therefore, the research directions of these two fields are merging.

Bioinformatics and Computational Biology involve the disciplines of Chemistry, Biochemistry, Molecular Biology, Genetics, Genomics, Molecular Evolution, Computer Science, Statistics and Mathematics. Input from these disciplines allows the results of Bioinformatics to be interpreted in many different ways. Research in Computational Biology often includes system biology.

Major research efforts in Bioinformatics and Computational Biology involve sequence alignment, gene annotation, genome assembly, protein structure alignment, protein structure prediction, prediction of gene expression, protein-protein interactions, the modeling of evolution and analysis of the mutations in cancer. In summary, research efforts have progressed from sequence alignment to finding sequence similarity, verifying gene annotation, observing protein structure, and then even further to analyzing mutations and modeling the evolution of genes.

Luscombe, Greenbaum, and Gerstein (67) proposed that “the aims of bioinformatics are three-fold.” The first aim is to provide the simplest way for researchers

to access and submit their produced work. The second aim is to “develop tools and resources that aid in the analysis of data.” The last aim is to use these established tools to analyze the data and give “results in a biologically meaningful manner.”

1.10 Introduction to Main Projects

This dissertation utilizes techniques from Computational Biology and Bioinformatics for gene recognition. The following sections briefly describe the main projects for this research.

1.10.1 Comparison of BLAST Software

The computational power required to search exponentially growing databases, such as GenBank (68), has increased dramatically. Several tools were developed in the past few years for rapid DNA and protein sequence comparison. One of the most widely used sequence alignment tools, known as BLAST (Basic Local Alignment Search Tool), was released in 1990 by the researchers at the National Center for Biotechnology Information (NCBI) (69). In 1995, one of the original authors of BLAST began development of the algorithm at Washington University (WU), independent of the NCBI effort. In the same year, the first release of WU BLAST (version 1.4) was released. Since the original development, a number of changes have been incorporated (70). In order to evaluate the effectiveness of BLAST for sequence comparison at a nucleotide level, a study was performed of three current versions of BLAST software: NCBI BLAST, WU BLAST and mpiBLAST.

In general, WU BLAST was found to be most efficient when the database and query sequences are unknown. NCBI BLAST appeared to work best when the database contains a small number of sequences, while mpiBLAST showed the power of database distribution when the number of bases per target database is large. The optimal number of compute nodes in mpiBLAST varies depending upon the database, yet in the cases studied, remained surprisingly low. A detailed description of the project will be discussed in Chapter 3, Sections 3.4 to 3.8.

1.10.2 Alternative Splicing Recognition

Studies in alternative splicing have found that a gene may produce more than one protein in order to fulfill different requirements and needs in cells. One gene with a large number of known splice variants is phosphodiesterase 4 (PDE4).

PDE4 is an enzyme that has been targeted for inflammatory disorders, including asthma, chronic obstructive pulmonary disease (COPD), and central nervous system (CNS) injury/disease. PDE4 has a large number of potential isoforms with apparent tissue and functional specificity. Potential roles of the different PDE4 isoforms within the CNS and other tissue types were studied using all four known rat PDE4 paralogs (PDE4A, PDE4B, PDE4C and PDE4D).

In order to see how ESTs can be used to detect novel isoforms and tissue specificity with alternative splicing for PDE4, a computational method to align rat ESTs to the corresponding chromosomal and mRNA sequences was developed. The splice pattern and tissue type for each EST was examined in order to gain more insight into

PDE4 isoform tissue specificity. Further discussion will be provided in Chapter 4, Sections 4.4 to 4.9.

1.10.3 Pseudogene Detection and Characterization

As genes provide the heredity information for an organism, pseudogenes are regions sharing sequence homology to the functional genes yet are unable to operate as genes. Pseudogenes can create difficulties in detecting genes *in silico*. A method to uncover pseudogenes and to differentiate between functional genes and pseudogenes is desired. Chapter 5 discusses an algorithm which was designed and implemented to detect and differentiate pseudogene.

1.11 Organization of Dissertation

The focus of this dissertation is to design and implement algorithms to analyze data computationally and the use of ESTs through three different but inter-related projects – comparison of current BLAST software in nucleotide sequences, alternative splice pattern recognition, and pseudogene detection and characterization. Chapter 2 introduces the necessary data components. Chapter 3 reviews the preprocessing methods for sequence alignment and examines the comparison of current BLAST software. Chapter 4 discusses the computational method for alternative splice pattern recognition and gives a case study in discovering rat PDE4 sequences. Chapter 5 details the methodology of pseudogene detection and characterization and provides a scoring scheme of distinguish genes and pseudogenes. Chapter 6 connects the three projects

discussed in Chapters 3 to 5 to draw conclusions regarding computational analysis for understanding gene regulation and provide ideas for extending the research in the future.

CHAPTER II

DATA SOURCES AND DATA PREPROCESSING

Since the invention of automated genome sequencers, a large amount of biological sequence data has been made available to the public. GenBank (68) was established in 1982 by the National Institutes of Health (NIH) to help advance scientific discovery worldwide. According to a news released from NIH, dated April 3, 2008, this comprehensive database has collected sequences from more than 240,000 organisms. The number of base pairs in GenBank doubles about every 19 months. Recently, the public collection of DNA and RNA sequences has reached approximately 110 million sequences and 200 billion base pairs (71). This data is maintained and exchanged on a daily basis under the International Nucleotide Sequence Database Collaboration (INSDC), which includes GenBank at the National Center for Biotechnology Information (NCBI), European Molecular Biology Laboratory (EMBL) and the DNA Data Bank of Japan (DDBJ). Every two months, a new version of GenBank is released. The three main sources of data for the three projects discussed in dissertation are ESTs, mRNA and genomic sequences from the GenBank and University California Santa Cruz (UCSC).

2.1 Sequence Data

2.1.1 dbEST

dbEST is division of GenBank that collects ESTs submitted from around the world. It contains sequence data and its annotation information from numerous organisms

(72). Newly discovered ESTs are submitted to dbEST daily. About every two weeks, dbEST releases an update on the number of entries. In addition, approximately every two months, GenBank releases a new version of flat format files which allow researchers to download the newest data to their workstations. Table 2.1 provides the top 25 organisms in GenBank/dbEST released on May 30, 2008

(http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). The total number of public entries in this release was 52,858,766. The human genome had the largest number of entries for all organisms (15.40%).

Table 2.1: Top 25 Organisms in dbEST (as of May 30, 2008)

Organism	# of entries
<i>Homo sapiens</i> (human)	8,137,747
<i>Mus musculus</i> + <i>domesticus</i> (mouse)	4,850,258
<i>Arabidopsis thaliana</i> (thale cress)	1,526,133
<i>Bos taurus</i> (cattle)	1,517,053
<i>Sus scrofa</i> (pig)	1,476,357
<i>Zea mays</i> (maize)	1,462,614
<i>Danio rerio</i> (zebrafish)	1,379,829
<i>Xenopus tropicalis</i> (western clawed frog)	1,271,375
<i>Oryza sativa</i> (rice)	1,220,876
<i>Triticum aestivum</i> (wheat)	1,051,465
<i>Ciona intestinalis</i> (sea squirt)	996,088
<i>Rattus norvegicus</i> + <i>sp.</i> (rat)	895,894
<i>Xenopus laevis</i> (African clawed frog)	677,784
<i>Oryzias latipes</i> (Japanese medaka)	609,699
<i>Gallus gallus</i> (chicken)	599,383
<i>Drosophila melanogaster</i> (fruit fly)	573,212
<i>Brassica napus</i> (oilseed rape)	567,177
<i>Hordeum vulgare</i> + <i>subsp. vulgare</i> (barley)	478,734
<i>Salmo salar</i> (Atlantic salmon)	433,337
<i>Glycine max</i> (soybean)	394,370
<i>Canis familiaris</i> (dog)	365,909
<i>Vitis vinifera</i> (wine grape)	352,984
<i>Branchiostoma floridae</i> (Florida lancelet)	346,107
<i>Pinus taeda</i> (loblolly pine)	334,502
<i>Physcomitrella patens subsp. Patens</i>	328,628

2.1.2 Reference Sequence (RefSeq)

The Reference Sequence (RefSeq) database (73-76) is a collection of annotated DNA, RNA and protein sequences from major organisms, including plasmids, organelles, viruses, archaea, bacteria, and eukaryotes. RefSeq contains data extracted from GenBank, and involves varying levels of computational and experimental verification from authoritative groups. Each RefSeq provides non-redundant information on genomic location, sequence and text annotation as well as genome annotation¹², gene characterization, mutation analysis, expression studies and polymorphic discovery. In addition, the database supports sequence standards for genomes and transcripts or proteins, providing a foundation for unambiguous comparison of functional information (76).

RefSeq is updated daily with new records added to the collection, sequence updates, and annotation changes. The May 2008 RefSeq collection (Release 29, dated 5-9-2008, <http://www.ncbi.nlm.nih.gov/RefSeq/>) was comprised of sequences from over 5,000 distinct taxonomies, ranging from viruses to bacteria to eukaryotes. It included whole chromosomes, organelles, plasmids, viruses, transcripts and more than 5,400,000 proteins. Table 2.2 shows the number of species in each sub-directory and the counts of accessions and length per type of molecule.

¹² Genome annotation is a process of assigning biological information to sequences. The process is based on two main steps: identifying elements on the genome and adding the biological information to the elements. An annotation is a note added by way of commentary and explanation. Once a genome is sequenced, it needs to be annotated and clarified.

Table 2.2: Statistical Information of RefSeq Sequences (as of May 9, 2008)

Species	# of organisms	Accessions		bp / residues
complete	5,168	genomic	1,510,537	102,394,850,748
fungi	99	RNA	1,382,728	2,276,250,402
invertebrate	353	protein	5,482,876	1,870,214,220
microbial	1,494	Abbreviations: #, number; bp, base pair		
mitochondrion	1,384			
plant	126			
plasmid	634			
plastid	131			
protozoa	87			
vertebrate (mammalian)	247			
vertebrate (other)	664			
viral	2,060			

2.1.3 UCSC Genome Browser

The University of California Santa Cruz (UCSC, <http://genome.ucsc.edu>) has created a variety of tools, including a graphical tool for viewing a specified region of a genome and a collection of aligned annotations (77-81). The database for the UCSC Genome Browser provides integrated sequence and annotation data for a large collection of vertebrate and model organism genomes, including human, mouse, rat, and fruit fly (Table 2.3). Each sequence in the UCSC Genome Browser database includes assembly data, comparative genome annotations, and mRNA, EST and RefSeq gene alignments from GenBank. The browser is updated whenever a revised genome is released. Beginning in 2006, UCSC publishes an annual article with information on the latest database, available tools for viewing and comparing genes and new features (77-81).

Table 2.3: Available Sequence and Annotation Downloads in UCSC Genome

Browser	
Verterbrates (Complete Annotation Sets)	human, cat, chicken, chimpanzee, cow, dog, fugu, guinea pig, horse, lamprey, marmoset, medaka, mouse, opossum, orangutan, platypus, rat, rhesus, stickleback, tetraodon, <i>X. tropicalis</i> (western clawed frog), zebrafish
Verterbrates (Sequences downloads only)	armadillo, bushbaby, elephant, european hedgehog, rabbit, shrew, tenrec, tree shrew
Deuterostomes	<i>C. intestinalis</i> (sea squirt), lancelet, <i>S. purpuratus</i> (purple urchin)
Insects	<i>A. gambiae</i> (African malaria mosquito), <i>A. mellifera</i> (western honey bee), <i>D. ananassae</i> , <i>D. erecta</i> , <i>D. grimshawi</i> , <i>D. melanogaster</i> (fruit fly), <i>D. mojavensis</i> , <i>D. persimilis</i> , <i>D. pseudoobscura</i> , <i>D. sechellia</i> , <i>D. simulans</i> , <i>D. virilis</i> , <i>D. willistoni</i> , <i>D. yakuba</i> , <i>T. castaneum</i> (red flour beetle)
Nematodes	<i>C. brenneri</i> (small nematode), <i>C. briggsae</i> (small nematode), <i>C. elegans</i> (nematode), <i>C. remanei</i> (worm), <i>P. pacificus</i> (roundworm)
Other genomes	<i>S. cerevisiae</i> (baker's yeast), SARS ¹³

2.1.4 Data Information

For this study, EST sequences were downloaded from GenBank/dbEST (72) at NCBI. The latest downloadable version was NCBI-GenBank Flat File Release 165.0 dated 4-15-2008 (<http://www.ncbi.nlm.nih.gov/dbEST/>). The mRNA sequences were downloaded from RefSeq (76) at NCBI. The latest release was NCBI Reference Sequence (RefSeq) Database Release 29 dated 5-4-2008 (<http://www.ncbi.nlm.nih.gov/RefSeq/>). It contained 5,168 organisms and 8,376,141 entries, including 1,510,537 genomic sequences (102,394,850,748 bases), 1,382,728

¹³ SARS, or severe acute respiratory syndrome, is a severe form of pneumonia which appeared in outbreaks in 2003. It is transmitted especially by contact with infectious material (as respiratory droplets or body fluids), and is characterized by fever, headache, body aches, a dry cough, hypoxia and usually pneumonia.

RNAs (2,276,250,402 bases), and 5,482,876 protein sequences (1,870,214,220 bases). Human and rat genomes were downloaded from the UCSC Genome Browser (<http://genome.ucsc.edu/>) (79). The latest release for the human genome was hg18 in 5-2004. The latest version of the rat genome was rn4 dated 11-2004.

2.2 Data Format

Sequences retrieved from the NCBI website can be displayed in several formats. These include ASN.1 (abstract syntax notation one) (82), FASTA (83), XML (extensible markup language) (84), and GenBank Flat File (85). Researchers can choose the specific format in order to receive the information they need. The two most common formats are GenBank and FASTA.

When a sequence is submitted to NCBI, it is recorded in the GenBank Flat File Format (85;86). The basic information for this format includes locus, definition, accession, version, keywords, source (a sub-category includes the organism), reference (sub-categories include authors, title, journal, medline, and pubmed), comment, features (sub-categories include source, coding region (CDS), gene), base count, and origin. Figure 2.1 shows a representative GenBank file for GenInfo Identifier (GI) 1392161.

SOURCE and LOCUS can be used to determine from which organism the sequences are derived and the size of the sequences. For a detailed description of each item involved in a GenBank file format, please refer to (85;86).


```

LOCUS      AA000001              474 bp    mRNA    linear    EST 17-OCT-1996
DEFINITION zd84h07.s1 Soares_fetal_heart_NbHH19W Homo sapiens cDNA clone
            IMAGE:347389 3', mRNA sequence.
ACCESSION  AA000001
VERSION    AA000001.1  GI:1392161
KEYWORDS   EST.
SOURCE     Homo sapiens (human)
  ORGANISM Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Catarrhini;
            Hominidae; Homo.
REFERENCE  1 (bases 1 to 474)
  AUTHORS  Hillier,L., Clark,N., Dubuque,T., Elliston,K., Hawkins,M., Holman
            ,M., Hultman,M., Kucaba,T., Le,M., Lennon,G., Marra,M., Parsons,J.,
            Rifkin,L., Rohlfing,T., Soares,M., Tan,F., Trevaskis,E., Waterston
            ,R., Williamson,A., Wohldmann,P. and Wilson,R.
  TITLE    The WashU-Merck EST Project
  JOURNAL   Unpublished (1995)
COMMENT    Contact: Wilson RK
            Washington University School of Medicine
            4444 Forest Park Parkway, Box 8501, St. Louis, MO 63108
            Tel: 314 286 1800
            Fax: 314 286 1810
            Email: est@watson.wustl.edu
            This clone is available royalty-free through LLNL ; contact the
            IMAGE Consortium (info@image.llnl.gov) for further information.
            Insert Length: 2259  Std Error: 0.00
            Seq primer: mob.REGA+ET
            High quality sequence stop: 403.
FEATURES   Location/Qualifiers
     source          1..474
                     /organism="Homo sapiens"
                     /mol_type="mRNA"
                     /db_xref="GDB:1272764"
                     /db_xref="taxon:9606"
                     /clone="IMAGE:347389"
                     /sex="unknown"
                     /dev_stage="19 weeks"
                     /lab_host="DH10B (ampicillin resistant)"
                     /clone_lib="Soares_fetal_heart_NbHH19W"
                     /note="Organ: heart; Vector: pT7T3D (Pharmacia) with a
                     modified polylinker; Site_1: Not I; Site_2: Eco RI; 1st
                     strand cDNA was primed with a Not I - oligo(dT) primer [5'
                     TGTTACCAATCTGAAGTGGGAGCGGCCGCATCTTTTTTTTTTTTTTTTTT 3'],
                     double-stranded cDNA was size selected, ligated to Eco RI
                     adapters (Pharmacia), digested with Not I and cloned into
                     the Not I and Eco RI sites of a modified pT7T3 vector
                     (Pharmacia). Library went through one round of
                     normalization to a Cot = 5. Library constructed by
                     M.Fatima Bonaldo. This library was constructed from the
                     same fetus as the fetal lung library, Soares fetal lung
                     NbHL19W."
ORIGIN
      1 taantgagat ctagggtatta acctgctgtc tagcgaaaac tagtcactaa gtcctggcct
      61 gagagatacc cacatttcct ttagaacaaa cagaactaat acctgtgtac atttctgaga
     121 gcttgatgtg tgagtcctta aaatgtagac cttgcaggag gcttagacct cagtttcacc
     181 taatgcatgt ggaggaaatg gaggtgagaa tagtcacctg aagagtgcac ggcgtccagc
     241 tccagcacac acactcttcc ctgggcagca ggaaaaggag gtaacaagga cttgggctga
     301 catctgaagc actangctaa tgtgcctggt agagggggagc ctcaggaagn cacaagatgg
     361 tcattccacc tngtagctgt ccacaaacct gaggtttcca catcgttttt aaagggcaca
     421 gtggggcaat gtgncaaggc agaaaaccaa taaccatttc aagggnctac ttgn

```

Figure 2.1: Example GenBank file 1392161.

A sequence in FASTA data format (83) begins with a greater-than symbol '>', followed by single line description. In strict FASTA format, the data includes the GI number, locus name, source organism, gene name/protein name, and molecular type. The symbol '|' is used to separate each piece information. The next few lines are sequence data, the combination of A, C, G and T nucleic acids or twenty different amino acids for that GI number. An example of the FASTA format for GI number 1392161 is shown in Figure 2.2. In a text file containing more than one sequence, the greater-than '>' symbol can be used to determine the beginning of the next sequence.

```
>gi|1392161|gb|AA000001.1|AA000001 zd84h07.s1 Soares_fetal_heart_NbHH19W Homo sapiens cDNA clone IMAGE:347389 3', mRNA
TAANTGAGATCTAGGTATTAACCTGCTGTCTAGCGAAACTAGTCACTAAGTCCTGGCCTGAGAGATACC
CACATTTTCCTTTAGAACAAACAGAACTAATACCTGTGTACATTTCTGAGAGCCTGATGTGTGAGTCCTTA
AAATGTAGACCTTGCAGGAGGCTTAGACCTCAGTTTCACCTAATGCATGTGGAGGAAATGGAGGTGAGAA
TAGTCACCTGAAGAGTGCAAGCGCTCCAGCTCCAGCACACACACTCTTCCCTGGCAGCAGGAAAAGGAG
GTAACAAGGACTTGGGCTGACATCTGAAGCACTANGCTAATGTGCCTGGTAGAGGGGAGCCTCAGGAAGN
CACAAGATGGTCATTCCACCTNGTAGCTGTCCACAAACCTGAGGTTTCCACATCGTTTTTAAAGGGCACAC
GTGGGCAAAATGTGNCAAGGCAGAAAACCAATAACCATTTCAAGGGNTCACTGN
```

Figure 2.2: Example FASTA format 1392161.

GenBank and FASTA file formats both provided the necessary information for this research project. However, FASTA files are much smaller than files in GenBank format due to the exclusion of various annotations. Once the appropriate data was downloaded, it was preprocessed for use in each project. Section 2.3 covers the necessary steps in data preprocessing.

2.3 Preprocessing Data

After the appropriate data was downloaded, it needed to be preprocessed. This involved filtering sequences by organism, masking repetitive elements and locating low complexity regions.

2.3.1 Filtering by Organism

Sequences in dbEST are submitted by researchers all over the world and organized into files according to the time they were received. Therefore, several different organisms may be represented in each of the dbEST files (release 165.0 included 713 files). Since the project examined ESTs on an organism-by-organism basis, the sequences had to be reorganized by organism before they could be analyzed.

Each sequence from the downloaded files was stored in GenBank flat file format (85). To re-organize the sequences by organism, the SOURCE field, which shows the scientific name of the organism, was used. A Perl script was written in order to read through the SOURCE field and assigned sequences to files according to organism.

A GenBank flat file format contains more annotation than is necessary for the purpose of sequence comparison. To align and compare two sequences, only the identifier and sequence data are needed. Therefore, a program called gb2fasta in the WU BLAST software package was used to convert the sequences into FASTA data format (83). After this conversion, the size of a sequence file was greatly condensed by removing annotations.

2.3.2 Masking Repetitive Elements

Raw ESTs which have been organized by organisms are not ready for sequence mapping and clustering. Repetitive elements are sequences that appear more than once in a genome. Most genomes contain repetitive elements. For instance, over 40% of the human genome is composed of repetitive elements (42;44). The presence of such sequences can severely lengthen the search time. Thus, these regions should be removed or masked from consideration within raw EST files.

RepeatMasker is a software package that was designed to screen DNA sequences for repeats (87;88). RepeatMasker compares input sequences to a library of repetitive elements including interspersed and internal repeats. For this study, repetitive elements were masked by hard masking, which replaces each of the nucleotides in a repetitive region with 'N'. Soft masking can also be used to replace the DNA characters with their lower case equivalent. The computational time for sequence comparisons is decreased after the repetitive elements are masked (57).

2.3.3 Filtering Low Complexity Regions

Low complexity regions are those "regions of biased composition including homopolymeric runs, short-period repeats, and more subtle overrepresentation of one or a few residues" (89). They occur in both nucleotide and protein sequences. A low complexity sequence is a sequence with a biased nucleotide or amino acid composition that differs significantly from the general composition observed in either the sequences or a dataset of sequences.

Masking these regions leaves biologically relevant regions available for matching and increases computational speed. The DUST (70) and SEG (70;90) programs are designed for this purpose. DUST is used especially for masking or filtering low-complexity regions in nucleic acids and SEG targets in amino acids. The low-complexity nucleic acid regions are masked with N's and amino acid regions are masked with X's.

CHAPTER III

COMPUTATIONAL SEQUENCE ALIGNMENT

There are many methods available for finding the percentage of similarity between two sequences. The two main types of alignment are global and local. Global alignment refers to aligning the entire length of two sequences. Local alignment looks for significant similarities in smaller sections of the sequences.

An exhaustive algorithm will search through all possible alignments to find the best match between two sequences. However, such an approach requires intensive computation. Even if the sequences are only 10 bases long each, there are $O(2^{10 \times 2^{10}}) = O(2^{10+10}) = O(2^{20}) = 1,048,576 \sim 1\text{M}$ comparisons. Therefore, more efficient approaches are needed and used.

3.1 Dynamic Programming Algorithm (DPA)

Dynamic programming is a method of solving the problems when the solutions can be determined recursively by building upon known solutions of a subspace. The term dynamic programming was first used by Richard Bellman in the 1940s (91). This type of problem has the properties of overlapping subproblems and optimal substructure. A famous example is finding the n th member of the Fibonacci sequence (See Equation 3.1). Later results are based on previous calculation.

$$fib(n) = \begin{cases} 0 & , if\ n = 0 \\ 1 & , if\ n = 1 \\ fib(n-1) + fib(n-2), & if\ n \geq 2 \end{cases} \quad (\text{Equation 3.1})$$

Dynamic programming can be used in sequence alignment by combining solutions from aligning sequence prefixes which are computed once and saved in a matrix (92). There are three steps in a basic DPA: matrix initialization, matrix scoring fill and traceback. A DPA can locate the minimal matching or mismatching bases and edit the distance between two strings by inserting or deleting gaps. DPA has an $O(m \times n)$ time and space complexity where m and n are the size of the strings.

Global alignment requires an alignment of one entire string X to a second entire string Y . Gaps may be inserted in either or both sequences to achieve the best result. The Needleman-Wunsch algorithm, which was proposed in 1970 by Needleman and Wunsch, is the most famous algorithm used to create a global alignment. It was the first instance of dynamic programming being applied to biological sequence comparison and it is guaranteed to find the alignment with the maximum score, given a scoring scheme (93).

Local alignment finds matching pairs of segments, each a part of a longer sequence, which have significant similarity. The Smith-Waterman algorithm, created in 1981, is one of the most famous algorithms used in local alignment (94).

In 1983, Ukkonen suggested a method to reduce computational complexity by adding an additional matrix in order to restrict the search space in the main matrix to a linear approach (95). In 1986, Myers used this approach and reduced the time to $O(nd)$ time in the worst case and $O(n+d^2)$ on average, where n is the length of the longer string of the two strings and d is the Hamming distance¹⁴ between the sequences. In addition, a penalty cost is included to deal with the gaps between two sequences (94;96).

¹⁴ If two sequences of length N differ from each other at n sites, then the proportion of differences, n/N , is referred as the Hamming distance or the degree of divergence.

3.2 Heuristic Algorithm

DPA will produce the optimal alignment for sequence comparisons. However, DPA is still computationally complex in terms of space and time. Therefore, a heuristic algorithm which “can make an approximation of the best solution without exhaustively considering every possible outcome (89)” like FASTA and BLAST, can reduce time and space complexity in calculation.

3.2.1 FASTA

FASTA stands for FAST-ALL. Developed by Pearson and Lipman, it combines local searching with a heuristic algorithm (97) to search a database for sequence homology. FASTA compares protein or nucleotide sequences to report the best matches of sequences for local alignment.

The FASTA program first generates a lookup table for matching sequence patterns of amino acids or nucleotides in a query sequence to sequences in a database without gaps. A parameter *ktup* is used, with a word size of 3-6 for nucleotide sequences and 1-2 for amino acid sequences, to screen the database for the pattern and insert matches into the lookup table. A scoring matrix is then used to determine the ten best aligned regions. Third, gaps are inserted between the high-scoring regions to join them together. Finally, a Smith-Waterman algorithm is used to align the highest scoring sequences. The optimal alignments of the query sequences to the best database matches will be printed as output (98-100).

3.2.2 BLAST

BLAST stands for Basic Local Alignment Search Tool. It is the most widely used software package for rapid DNA and protein sequence comparison. It can be used to detect high scoring areas of local similarity between a query sequence and a target database of sequences.

BLAST searches sequences one-by-one in the database. If the current sequence in the database shares similarity to the query, BLAST returns the result to the user. Otherwise, BLAST will search the next sequence in the database.

The initial filter of the BLAST algorithm searches for seed sequences of a particular length, 11 bases for NCBI nucleotide-nucleotide BLAST, with 100% conservation between the target and query sequences. Initial hits of seed sequences are then extended to check for larger regions of similarity. Through these searches, BLAST makes it possible to look for sequence homology within sequences by performing database searches, motif searches, and gene identification for both DNA and protein sequences.

There are two versions of BLAST used widely in research. One is free, the version produced by the National Center of Biotechnology Information (NCBI), called NCBI BLAST. The other requires an academic license agreement. This is the version created by Washington University (WU) in Saint Louis, Missouri, called WU BLAST (69;70).

3.2.3 Current BLAST Tools

The BLAST software was first published in 1990 by researchers at the National Center of Biotechnology Information (NCBI). In the nearly 18 years since its original development, a number of changes have been made. As mentioned previously, the most widely used versions of BLAST are NCBI BLAST and WU BLAST. Both versions of BLAST offer a suite of programs. These programs perform nucleotide-nucleotide comparison (`blastn`), protein-protein comparison (`blastp`), nucleotide-protein comparison (`tblastn`), and translated nucleotide-nucleotide comparison (`tblastx`).

In addition, the NCBI suite offers two programs for looking at patterns gathered from multiple alignments of sequence conservation as seeds into sequence alignment. These two programs are position specific iterated BLAST (PSI-BLAST) and pattern hit initiated BLAST (PHI-BLAST) (101;102).

Speed is a problem in searching large quantities of DNA and protein sequences, even with heuristic algorithms such as BLAST. However, BLAST has qualities that lend it to being parallelizable.

3.2.4 mpiBLAST

An open source of parallelization of BLAST was developed as mpiBLAST by the Los Alamos National Laboratory and released at the High Performance Computing Revolution 2003. This tool attempts to reduce disk I/O in a single machine and parallelize NCBI BLAST on PC clusters.

mpiBLAST splits a large database into smaller fragments and performs the master-worker scheme of job running. Through the technique of message passing

interface (MPI) (103;104), mpiBLAST sends out sequence database fragments to different nodes. Each node individually performs the computation of NCBI BLAST on the query sequence and assigned fragment of original database. When all requested nodes finish their sub-tasks, the master node combines the results and send it back to the job sender (105;106).

3.3 EST to Genome Alignment Algorithms

Several programs align ESTs to genome sequences. Each of them has its own purpose. For example, sim4 is designed to quickly align a cDNA sequence to its parent sequence with high accuracy (107) while Spidey aligns cDNA sequences without considering the length of introns (108). Compared to more commonly used tools like BLAST, these programs incorporate more specific goals of distinguishing the boundaries of exons and introns.

Specific nucleotide pairs, such as the canonical CT/AG and GT/AG (109-113), are usually found at exon/intron boundaries. est2genome, Spidey, sim4, GMAP and EXALIN can help to find the positions of exon/intron boundaries, for fine tuning the alignments.

3.3.1 est2genome

est2genome is a tool used to predict genes by finding sequence homology. It implements a linear space dynamic programming algorithm to compare spliced sequences with their genomic counterparts on both the forward strands and reverse complement. est2genome will report a list of the exons and introns and indicate the predicted gene transcription (114).

3.3.2 Spidey

Spidey was designed in 2001 to accomplish two main goals: “finding good alignments regardless of intron size” and “avoiding getting confused by nearby pseudogenes and paralogs” (108). It uses BLAST and a DPA type dot-plotting method (115) called DotView to align a single genomic sequence to a group of mRNA sequences. The hits resulting from these local alignment tools are sorted and assigned into different windows. Another round of BLAST is performed with the entire mRNA sequences in each window. A summary of the alignment is reported.

3.3.3 sim4

The program sim4 aligns a cDNA sequence with another genomic sequence rapidly and accurately (107;116). It starts with a BLAST-like computation to find high-scoring pairs (HSPs) of aligned regions between two sequences. Next, sim4 uses DPA to choose a set of HSPs which might represent a gene in increasing order. sim4 allows for introns in the calculation, but the introns must be distinguishable in the results. Therefore, sim4 determines the boundaries of exons and computes the alignments (107).

3.3.4 GMAP

GMAP stands for Genomic Mapping and Alignment Program. It aligns mRNA and EST sequences to genomic sequences. Before processing GMAP, the data is entered into an indexed database file. Then GMAP finds and reports the location of an EST, aligns the EST to genomic sequences, and provides an integer interval tree to store the look up information associated with a given cDNA’s position in the genome. It can map

and align a cDNA over multiple genomic sequences at the same time. However, GMAP does not provide statistical analysis of alignments and therefore users cannot filter results based on statistical significance (117).

3.3.5 EXALIN

EXALIN, which was released in 2006, compares sequences one by one. Its approach is based on a novel method for searching spliced alignment, comparing sequence similarity with that obtained from position specific scoring matrix (PSSM) splice site models. In order to maximize the power of discrimination in sequence alignment and comparison, the scoring systems are chosen based on genomes. Currently, the scoring matrices and splice site models for human, mouse, rice and *Caenorhabditis elegans* are available. Dynamic programming was implemented in the tool to find the optimal computational result. Although dynamic programming returns the best result in alignment, it is slow in computation. Therefore, input output from `blastn` as an optional choice in EXALIN would help speed up the comparison (118).

3.4 Case Study in Comparison of Current BLAST Software

Sections 3.2.2 to 3.2.4 described how BLAST works. NCBI BLAST, WU BLAST and mpiBLAST all have a level of parallelization built in. The default setting of NCBI BLAST is to run on a one processor using multithreads. WU BLAST runs two threads¹⁵ at the same time in one processor machine. mpiBLAST requires at least two nodes, but can run on an unlimited number depending on the number of processors available.

¹⁵ A thread is a portion of a program that can run independently of and concurrently with other portions of the program.

Theoretically, searching for sequence similarities in a parallel fashion on multiple machines should be faster than using a single processor on the same data set. With this pre-assumption, mpiBLAST should be the fastest of the three BLAST programs.

As discussed in Section 1.10, ESTs are used to detect novel isoforms and tissue specificity for alternative splicing events. In addition, ESTs are used to identify the possible locations of pseudogenes in chromosomal sequences. All of these processes start with alignment of ESTs to the genome. Therefore, a study was conducted to compare the efficiency of each version of BLAST software: ncbiBLAST, wuBLAST and mpiBLAST. Finding the most efficient BLAST algorithm would speed up the initial alignment of ESTs to genome and reduce the computational time of the following projects.

3.5 Methods

3.5.1 Data

Comparisons were made by focusing on human EST and genomic data. Thirteen representative human EST sequence files and five representative chromosome sequence files were chosen (Table 3.1). Each sequence file contained data for multiple sequences. These files were chosen based on the total number of sequences in the file, the total number of nucleotide bases in the file and the average sequence length to ensure that they were representative snapshots of the entire human EST and genomic data set.

Table 3.1: Data Used for BLAST Comparison

	Sequence Name	File Size	No. of Sequences	No. of bases
Genome	chr14	88,935,052	1	87,191,216
	chr22	35,039,241	11	34,352,072
	chr17	80,964,851	30	79,346,966
	chr9	120,146,880	60	117,790,386
	chr1	231,366,553	91	226,828,929
EST	est283	3,350	7	2,593
	est277	7,259	15	5,781
	est136	300,995	521	210,054
	est147	724,214	1,085	563,737
	est149	1,603,440	2,433	1,244,897
	est126	2,466,032	3,031	2,053,889
	est241	3,312,648	4,991	2,502,256
	est176	7,002,704	10,149	5,274,323
	est113	19,434,153	20,347	16,714,000
	est270	16,447,012	24,764	12,934,924
	est1	19,969,430	36,800	14,021,420
	est91	33,883,614	50,522	27,683,871
	est166	33,064,286	71,748	25,130,960

Abbreviations: chr, chromosome; No., number.

Chr1 represents chromosome 1; est1 represents dbEST file 1.

Table adapted from Table 1 in (119).

EST sequence data was downloaded using the GenBank Flat File Release 141.0, dated 4-15-2004 (72). The resulting 305 dbEST files were parsed to include only those with *Homo sapiens* as the organism. After the human ESTs were identified, they were masked for repetitive elements using RepeatMasker (87;88).

The repeat masked human goldenpath genomic assembly hg16-Jul2003¹⁶, dated 7-2003, was downloaded for use from UCSC Genome Browser (<http://genome.ucsc.edu>)

¹⁶ The genomic sequences in goldenpath genomic assembly hg16-Jul2003 version were split in pieces in most chromosome in 2003 because the sequences were still under debugging process. In addition, the technique to save contents in a text file has its upper limit in file size. The latest version of hg18-Mar2006 has merged most contigs from the same chromosome into one complete sequence. Those pieces that are not clear defined are placed in the random sequence. Therefore, for most chromosomes in the Human Genome would have up to two sequences in file.

(77-81). Each contig¹⁷ for a particular chromosome was placed into a file based on its cytogenetic position as an individual sequence entry. Blastable databases for each chromosome were created using the appropriate tool (xdformat for wuBLAST; formatdb for ncbiBLAST; and mpiformatdb for mpiBLAST) (92;106).

For mpiBLAST, the database was also segmented into fragments using the command mpiformatdb. Each fragment might not contain the same number of sequences, but should be nearly equal in overall size. In order to determine the number of fragments to use in mpiBLAST, three different sequences, chr22, chr1 and est166 were examined. The number of fragments, and therefore nodes used, was determined according to the resulting fragment size. The results are shown in Table 3.2.

Table 3.2: Fragment Information in mpiBLAST

sequence	No. of fragments	size per fragment
chr22	9	~1MB
	8	~2MB
	5	~4MB
	3	~9MB
	1	~35MB
chr1	44	~2MB
	30	~5MB
	25	~8MB
	20	~11MB
	15	~19MB
	10	~19MB
	1	~227MB
est166	26	~1MB
	13	~2MB
	7	~4MB
	1	~26MB

Abbreviations: chr, chromosome; No., number

Table adapted from Table 2 in (119).

¹⁷ A contig is a continuous sequence of DNA created by the assembly of overlapping sequenced fragments of a chromosome. It is also a group clones representing overlapping regions of a genome.

3.5.2 Types of Sequence Searches

The factors affecting speed are the size of the database, the size of the query, the number of database sequences and the number of query sequences. Types of sequences searches can be:

- A single, relative short sequence (such as a single EST) vs. a larger database;
- A larger database of smaller sequences (with different lengths) vs. a large database of smaller sequences;
- A large database of smaller sequences vs. a large database of large sequences;
- A large database of large sequences vs. a large database of larger sequences.

In order to determine the efficiency of the various versions of BLAST, the representative EST and genomic data were used in four separate comparisons between different target database (T) and query sequences (Q): EST (T) vs. EST (Q); chromosome (T) vs. chromosome (Q); chromosome (T) vs. EST (Q); and EST (T) vs. chromosome (Q).

EST vs. EST compared the computational efficiency when sequences in both the database and query are short. Chromosome vs. chromosome tested for situations when both query sequences and target databases are large sequences. Compared to an EST sequence, which contains only 300-600 bp on average, the number of nucleotide bases in a chromosome contig is much larger. However the number of sequences in each chromosome file is smaller, ranging from one sequence (chr14) to 91 sequences (chr1) for the representative sequences. Therefore, in chromosome vs. EST, the longer (in base length) sequences, or ESTs, were used as a database and the smaller sequences were used as the query. In the fourth comparison, we reversed the database and query sequences as

EST vs. chromosome in order to determine if the order of comparison had an impact on computational results.

3.5.3 System and Software

The comparisons were based on the current version of the appropriate BLAST software available at the time of analysis. ncbiBLAST 2.2.9 (release date 5/12/2004) was downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). For wuBLAST, an academically licensed version 2.0MP WashU (release date 8/22/2004) was downloaded from Washington University BLAST Archives (<http://blast.wustl.edu>). mpiBLAST 1.2.1 (release date 2/6/2004) was downloaded from mpiBLAST¹⁸.

In order to test the parallel versions, a 16-node (32 CPU) Microway Athlon MP GigaCluster™ with an additional master node was used. Each node on the cluster has a dual AMD Athlon™ MP 2400+ 384KB cache, 512 MB DDR, 266 MHz ECC/REG low profile, plus a 40GB hard drive. Red Hat Linux version 7.3 (120) with MPICH (103;104), Portable Batch System (PBS) (121), and Microway Cluster Management Software were installed. wuBLAST, ncbiBLAST and mpiBLAST were run on three different systems. Each job was sent and managed by PBS.

wuBLAST and ncbiBLAST were also compared using two identical PCs. These PCs were equipped with a dual AMD Athlon™ MP processor 2800+ 512KB cache. These machines also had a 640KB system RAM, a 2048 Extended RAM and a 120GB mass drive as a storage system. The operating system was Suse Linux version 9.1 (122), 2.6.4-52-smp i686 machine, and i386 hardware platform.

¹⁸ The mpiBLAST website used for this study was hosted by the Los Alamos National Laboratory (<http://mpiblast.lanl.gov>) at the time the project was developed. Since then two of mpiBLAST's main authors moved to Virginia Tech, the mpiBLAST website has been changed to <http://www.mpiblast.org/>.

3.6 Results

3.6.1 EST vs. EST

The first series of comparisons involved short length sequence database versus short length sequence queries. All thirteen representative EST sequence files (Table 3.1) were placed into individual blastable databases. Each database was aligned to the thirteen representative EST sequences files one at a time.

The test revealed that when the number of sequences in the database is small, ncbiBLAST seems to perform marginally better than wuBLAST (Table 3.3). The results indicated a delay of up to ~690 sec (~11.5 min) for wuBLAST on the data used.

However, as the number of sequences in the database increased, the processing time for wuBLAST tended to be smaller in comparison to ncbiBLAST (Table 3.4). This is especially evident in the four comparisons of the last two sequences, est91 vs. est91, est91 vs. est166, est166 vs. est91, and est166 vs. est166, where it took wuBLAST 3,699~7,702 seconds (61.2~128 minutes) to complete the work while it took ncbiBLAST 8,557~14,312 seconds (142.6~238.5 minutes), or approximately twice as long, to do the same job.

It took more time complete each task for est270, which contained 24,764 sequences, especially using wuBLAST. When est270 served as a query, it required more time than the other three longer sequences: est1, est91, and est166. When est270 was used as both the query and database, it took 12,822 seconds (237 minutes) to perform the comparison. This made the cumulative time to complete the whole series higher than any other series in Figure 3.1.

Table 3.3 ncbiBLAST Performs Best when the Number of Sequences in the Database is Small (Measured in sec)

query	# of sequence	database													
		est283		est277		est136		est147		est149		est126		est241	
		wu	ncbi	wu	ncbi	wu	ncbi	wu	ncbi	wu	ncbi	wu	ncbi	wu	ncbi
est283	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0
est277	15	0	0	0	0	0	0	0	0	0	0	0	0	0	1
est136	521	10	5	10	5	15	10	16	11	18	14	22	14	21	18
est147	1,085	21	10	21	12	27	20	35	28	41	47	41	35	52	70
est149	2,433	47	23	47	25	59	44	71	62	98	118	88	77	120	175
est126	3,031	59	30	60	33	77	58	82	67	104	87	132	115	124	135
est241	4,991	96	48	97	53	116	91	142	127	199	232	176	162	244	364
est176	10,149	197	98	197	108	262	189	299	236	387	402	414	321	460	573
est113	20,347	395	212	402	226	492	384	535	425	629	505	771	686	725	742
est270	24,764	472	241	485	259	749	436	758	496	965	691	1430	769	1302	947
est1	36,800	693	344	698	369	834	615	910	709	1071	975	1238	1014	1341	1325
est91	50,522	959	495	970	527	1242	888	1253	1025	1435	1263	1697	1544	1820	1777
est166	71,748	1338	670	1394	707	1587	1149	1690	1372	1897	1760	2189	2057	2312	2373

Table 3.4: wuBLAST Performs Best when the Number of Sequences in the Databases is Greater (Measured in sec)

query	# of sequence	database											
		est176		est113		est270		est1		est91		est166	
		wu	ncbi	wu	ncbi	wu	ncbi	wu	ncbi	wu	ncbi	wu	ncbi
est283	7	1	1	1	1	1	1	1	1	1	2	1	2
est277	15	1	1	1	1	1	1	1	2	2	2	2	3
est136	521	43	28	60	43	175	44	66	50	88	74	63	74
est147	1,085	79	100	99	109	190	114	122	124	163	187	137	186
est149	2,433	168	242	212	234	385	252	247	277	337	417	295	406
est126	3,031	226	194	378	357	749	366	368	349	584	590	435	585
est241	4,991	329	496	430	488	756	526	502	574	676	860	601	847
est176	10,149	890	1021	1158	1014	3160	1067	1251	1136	1793	1749	1364	1754
est113	20,347	1207	1101	2860	2789	4064	2314	1980	2148	3729	4240	2642	3836
est270	24,764	3475	1451	3565	2834	12822	3384	5114	2614	6911	4214	4341	4236
est1	36,800	2078	2003	2785	3114	5307	3237	3446	3720	4203	5424	3781	5443
est91	50,522	2800	2607	4722	5021	7227	4846	4343	4928	3669	9882	5772	8557
est166	71,748	3109	3653	4857	6319	6978	6362	5316	6613	7727	11210	7702	14312

Figure 3.1: Cumulative results of wuBLAST and ncbiBLAST in EST vs. EST comparison.

(283: est283; 277: est277; and so on) Image adapted from Figure 2 in (119).

After scanning the sequences in the est270 file, one possible reason that it took longer to complete, with the longest time occurring in comparisons of est270 with itself in wuBLAST, is that there are poly-A tail sequences in the file. This kind of sequence can waste a lot of computation time. If a low-complexity filter, like DUST (70) or SEG (70;90), was utilized, this type of sequence could be masked or skipped, allowing the program to proceed to the next comparison. However, when the test was run, both wuBLAST and ncbiBLAST were operating by the default setting. In ncbiBLAST, low-complexity filter DUST is turned on by default for the `blastn` program and SEG is turned on by default for the `blastp` program. But in wuBLAST, users must add an additional command of “`filter=dust`” to activate the low-complexity filter. This

would explain why wuBLAST took significantly more time than ncbiBLAST to process the est270 vs. est270 comparison. The wuBLAST program was attempting to compare the low-complexity sequences.

To run an mpiBLAST search, the minimum size of a database fragment is 1MB. Therefore, the chosen EST sequence files under 1MB could not be tested in mpiBLAST because they could not be segmented into more than one database fragment.

The results for mpiBLAST using an EST vs. EST comparison are shown using est166 as the database, and four of the representative EST sequence files as the query (est136, est147, est241 and est176). All mpiBLAST searches were tested using 1-15 nodes, excluding the master node, due to the upper limitation of having 16 nodes on the system. The results indicated that as the number of nodes used to complete the search increase, the total search time decreased (Figures 3.2 and 3.3). However, mpiBLAST did not appear to be more efficient at searching for sequence similarities.

Figure 3.2: est166 target database vs. est136 and est147 query sequences.
Image adapted from Figure 3a in (119).

Figure 3.3: est166 target database vs. est241 and est176 query sequences.
Image adapted from Figure 3b in (119).

3.6.2 Chromosome vs. Chromosome

It is possible the performance would vary drastically when both the target database and query contain a large number of bases per sequence due to a high memory load and increased disk I/O. In order to test this hypothesis, all five chromosome contig files were placed into individual blastable databases. Each database was compared to the five representative chromosome sequences separately.

Twenty-five comparisons were generated and the four cumulative results are presented in Figure 3.4. As the number of nucleotide bases, or size of the sequences increased, the cumulative time for both wuBLAST and ncbiBLAST to complete the series increased proportionally. In general, ncbiBLAST took 1.7~2.8 times longer to complete the series of work for each chromosome than wuBLAST. The cumulative time to complete the whole series of comparisons for chr1 as the database in wuBLAST was 53,301 seconds (~14.8 hours) (result not shown). It took almost a month to perform the same series of comparisons in ncbiBLAST. The reason for the tremendous increase in time in ncbiBLAST for chr1 is unknown, but could be related to a heavy memory load caused by the large number of sequences (91) and bases (226,828,929).

Figure 3.4: Cumulative results of wuBLAST and ncbiBLAST using chromosome vs. chromosome.

Image adapted from Figure 7 in (119).

3.6.3 Chromosome vs. EST

Comparing chromosome to EST uses larger sequences as target databases and smaller sequences as queries. The gain in efficiency of running mpiBLAST instead of ncbiBLAST appeared after using chromosome sequences as the database. The results for mpiBLAST were derived by using chr22 and chr1 as databases and two representative EST sequences, est136 and est147, as the queries. The results from wuBLAST, ncbiBLAST and mpiBLAST are shown in Figures 3.5 and 3.6, with a variable number of nodes used for mpiBLAST (as listed in the figure legend).

Figure 3.5: chr22 target database vs. est136 and est147 query sequences.
 Image adapted from Figure 6a in (119).

Figure 3.6: chr1 target database vs. est136 and est147 query sequences.
 Image adapted from Figure 6b in (119).

While mpiBLAST ran on multiple nodes in the cluster, wuBLAST and ncbiBLAST ran on a single node in the same cluster. The database is divided into 1,3,5,8, and 9 segments for chr22 and 1,10,15,20, 25, 30 and 44 segments for chr1 (Table 3.2). These numbers are listed as mpiBLAST-number_of_segments. The number of nodes used to complete the task is listed in the parentheses.

wuBLAST worked the best. The speed of mpiBLAST increased when the database was divided into more than 1 segment. However, if the database had only one segment, mpiBLAST performed slightly worse than ncbiBLAST due to the time it took for the master-worker scheme to deliver commands. It is worth noting that communication overhead for mpiBLAST has a significant impact on the database for searches with one segment.

3.6.4 EST vs. Chromosome

Comparisons using smaller sequences for the database and larger sequences as queries are unlikely in real world situations. However, in order to determine if the order in which these comparisons are made makes a difference on software performance, EST vs. chromosome was studied.

As in EST vs. EST, all thirteen representative EST sequence files were placed into individual blastable databases. Each database was searched for similarity to the five representative chromosome sequence files consecutively.

Each database search was performed as a series of five queries. Figure 3.7 shows the comparison between wuBLAST and ncbiBLAST as the cumulative time to finish each series of searches.

Figure 3.7: Cumulative results of wuBLAST and ncbiBLAST for EST vs. chromosome.

(283: est283; 277: est277; and so on) Image adapted from Figure 4 in (119).

Generally speaking, wuBLAST performed better than ncbiBLAST. ncbiBLAST took 1.6~4.5 times longer than wuBLAST to accomplish each series of comparisons.

The cumulative time taken by ncbiBLAST increased proportionally to the number of sequences in the database. This was not the case for wuBLAST. For instance, est270 took less time for wuBLAST to process than est113. According to Table 3.1, the reason might be that est270 has more sequences (24,764) but bases (12,934,924) than est113 (20,347 sequences, 16,714,000bp). Therefore the average number of bases per sequences is smaller. In another example, est166 was processed faster than est91.

3.6.5 mpiBLAST

Figure 3.8 shows the results obtained by using different numbers of database fragments segmented for mpiBLAST with est166 as the database and est136 as the query. Theoretically, the relationship between the number of nodes and the speed of computation should be linear, as long as the number of fragments does not exceed the number of available processors. For example, for the thirteen fragments in Figure 3.8, the result of running on two nodes should be half of the result of running on one node. Likewise the result of running on three nodes should be one third of the result of running on one node, and so on. Thus, running thirteen nodes should produce the best result because there are thirteen fragments. However, as Figure 3.8 illustrates, dividing the database into seven fragments and running on three to four nodes provided the best result. If the database was divided into either thirteen or 26 fragments, the best results were obtained by running on six to seven nodes. For a single database fragment, the number of nodes did not make difference, as Figure 3.9 illustrates.

One notable exception is that the time for 26 fragments dropped when thirteen nodes were used in cluster. $26 \div 13 = 2$. The reason might be that equal number of fragments were routed to the worker nodes so that each node ran balanced jobs. Moreover, when the chromosome was used as the database, the optimal number changed (Figure 3.9).

Figure 3.8: est166 target database vs. est136 query sequence in mpiBLAST.
 Image adapted from Figure 8a in (119).

Figure 3.9: chr1 target database vs. est136 query sequence in mpiBLAST.
 Image adapted from Figure 8b in (119).

3.7 Discussion

As mentioned in Section 3.6.1, the comparisons were made using the default settings for the BLAST tools. In general, wuBLAST worked the best, if we do not consider the number of bases in a sequence and the size of the database. What causes wuBLAST to run faster is difficult to examine because it is not open source.

For biologists, ncbiBLAST is considered standard because it was the first of the BLAST tools developed for sequence comparison. However, this study suggests wuBLAST is worth the extra licensing fees and installation effort.

The average number of bases per sequence for each chromosome was much larger than the average number of bases per sequence for the ESTs. Hence, if both the size of a chromosome fragment and the size of an EST fragment was ~1MB, the chromosome fragment would contain fewer sequences with a larger average size than the EST fragment.

`blastn` compares sequences one by one between the database file and the queries. Fewer similarities between the database and query will result in a smaller time requirement for the comparison.

`mpiBLAST` attempts to decrease disk I/O. It performs better than `ncbiBLAST` because the content of each fragment can easily fit into cached memory. It avoids the allocation problem of large contents in memory causing more disk I/O. However, sending the database fragments to different nodes to complete the whole task required time for communication between the master node and slave/worker nodes. Figures 3.5, 3.6, 3.8 and 3.9 indicate where potential bottlenecks occurred. Another consideration is network

flow. A network bottleneck could be a problem if lots of data needed to be transported at the same time.

In addition, breaking the database into pieces could affect the E-value. It would not be the same as the E-value calculated at once in any singular BLAST. Moreover, mpiBLAST itself does not change any I/O method to speed up its calculation. It first passes the database fragments to the slave/worker nodes and calls ncbiBLAST to run in parallel on those worker nodes.

3.8 Conclusion

To help identify any organism's transcriptome or transcribed portion of the genome, clustering a large number of sequences is necessary. It requires not only thousands of comparisons, but rather searching billions of records. Therefore, finding the best technique to compare sequences in parallel becomes paramount.

Based on the results, wuBLAST generally performs the best of the three tools using the default settings. However when dealing with a smaller number of sequences in the database, ncbiBLAST performs better. mpiBLAST decreases search time when the number of sequences in a fragment is small but the number of nucleotide bases is large. The user can choose a suitable BLAST based on the size of the data and the number of sequences. Note that these results are based on nucleotide sequences. There could be a different result in comparison of amino acids/protein sequences.

CHAPTER IV

COMPUTATIONAL METHOD FOR ALTERNATIVE SPLICE PATTERN RECOGNITION

4.1 Introduction

4.1.1 History of Alternative Splicing

As discussed in Section 1.6, a DNA sequence is translated into a fully-functioning protein through transcription, pre-mRNA processing, translation and protein folding (Figure 4.1). During this process, the cellular machinery may splice out an exon or retain an intron in whole or in part before producing the final copy of mRNA transcript, resulting in the synthesis of a greater variety of proteins. This is called alternative splicing. Exons of the primary gene transcript are separated and then reconnected to produce an alternative ribonucleotide arrangement.

Figure 4.1: Central Dogma.

The mechanisms for alternative splicing were first uncovered during the late 1970s and early 1980s. In 1977, Phillip A. Sharp of the Massachusetts Institute of Technology (MIT) and Richard J. Roberts of New England Biolabs discovered that in the primary RNA transcripts, the non-coding regions – introns – were discarded for the final

version of mRNA (123;124). Sharp and Roberts received a joint Nobel Prize in Medicine in 1993 “for their discoveries of split genes.” In 1980, Randolph Wall of University of California Los Angeles (UCLA) showed that this basic view of pre-mRNA splicing, in which the introns are always discarded and all exons are always included in the mRNA, does not invariably hold true. In fact, the cellular machinery can “decide” to splice an exon or to leave an intron in the final mRNA transcript (125). In 1984, Tom Maniatis, Michael Green and their colleagues at Harvard University developed a test-tube procedure to reveal the molecular machinery performing the cutting of introns and pasting together of exons (126-128).

4.1.2 Splicing Mechanism

The biological mechanism which controls splicing was first described in 1984 as a splice machine now commonly known as a spliceosome (126). The spliceosome is a complex of five small nuclear uridine rich (snRNA¹⁹) molecules labeled U1, U2, U4, U5 and U6 along with protein factors. Spliceosomes recognize exon/intron boundaries through a 5' donor site, a 3' acceptor site, a branch point and a polypyrimidine tract.

The canonical donor site recognized by the spliceosome complex is marked by the nucleotide GT, while the canonical 3' acceptor site is AG. There are other patterns confirmed within mammalian genomes including GC/AG, AT/AC, GT/GG and TT/AG (129;130). The canonical pattern GT/AG is predicted to occur 99.24% of the time. GC/AG occurs 0.69%, AT/AC occurs 0.05%, and the other patterns account for the remaining for 0.02% of all splice sites in mammals (129;130). The pattern AT/AC has

¹⁹ A snRNA is a small RNA molecule that functions in the nucleus by guiding the assembly of macromolecular complexes on the target RNA to allow site-specific modifications or processing reactions to occur.

been shown to result from a separate spliceosome complex involving U11 and U12 snRNA.

When canonical splicing begins, the U1 snRNA binds to the 5' end of the splice site and recruits several protein factors to form a commitment complex (131-133). This creates a branch site in which exons can attach at the donor's G nucleotide through a phosphodiester linkage. As part of the process, the U2 snRNA binds to the branch site. Afterwards, the U4-U5-U6 complex arrives and U6 replaces the U1. U1 and U4 disassociate from the complex. U2 and U6 link with the lariat intron that gets removed. U5 aids in merging the two adjoining exons together. Figure 4.2 shows the diagram of a canonical spliceosome.

Figure 4.2: Canonical spliceosome for intron splicing.

4.1.3 Alternative Splicing Modes

There are a number of alternative splicing events, including: alternative promoter selection, alternative polyadenylation sites, exon skipping, exon retention, exon extension, exon truncation and intron retention (Figure 4.3). Each of these is discussed in detail.

Figure 4.3: Alternative splicing events.

1. Alternative promoter selection

In alternative promoter selection, alternative aminyl ends are produced in the processed mRNA by alternatively selecting promoter regions. These regions occur in the 5' untranslated region (UTR) of the mRNA. Such events do not alter the final protein product. This is the only method that can produce an alternative N-terminus domain in proteins. Different sets of promoters can splice certain sets of other exons.

2. Alternative polyadenylation sites

In some cases, the 3' UTR of the processed mRNA is alternatively spliced producing alternative polyadenylation sites. Once again, these events will not alter the translated protein product.

3. Exon skipping

This method of alternative splicing works by choosing certain exons to remove from the final processed mRNA. As a result, each exon can be thought of as a “cassette” that can potentially be removed. Exon skipping results in an altered translated proteins (134-136). This is the most commonly found version of alternative splicing in mammals.

4. Exon retention

In exon retention, additional exons located in the place that is normally an intronic region, are added to the final mRNA sequences, resulting in a different translated protein (137).

5. Exon extension

In exon extension, sequences are added around a cassette exon at either the 5' or 3' ends. This results in a slightly modified mRNA sequence and produces a different protein (134;135;138).

6. Exon truncation

Exon truncation occurs when a cassette exon is not fully added to an mRNA sequence. The 5' end, 3' end or both ends of the exon are removed, resulting an altered translated protein (137).

7. Intron retention

This method of alternative splicing occurs when an intron is retained in the final mRNA transcript (134;135;138). As long as an expressible stop codon is not located in the intron, an altered translated protein is produced which is still functional. Intron retention is commonly found in plants and in lower multicellular organisms.

4.1.4 Characteristics of Alternative Splicing

Alternative splicing results in transcriptome/proteome complexity and gene function diversity. It is also highly relevant to human diseases. For instance, the breast cancer gene BRCA2 is a typical example of an exon skipping event (139). In addition, alternative splicing alters important protein features, such as phosphorylation, glycosylation, and transmembrane helices (140).

4.1.5 Rates of Alternative Splicing

Initially, alternative splicing was thought to be an anomaly (125). In the early 1990s, it was predicted that about 5% of all human genes provided alternatively spliced isoforms (124). By the late 1990s, researchers concluded that alternative splicing is much more prevalent than previously thought, with the potential for 50% of human genes to undergo alternative splicing (141;142). In recent studies, it has been found that up to 74% of human genes are alternatively spliced (143).

The average mammalian gene has between seven and eight exons (42). While this number seems small, this provides the possibility of over two hundred different cassette exon patterns. The largest number of known isoforms for a single coding region is the *Drosophila* DScam gene which has 38,016 distinct forms (144).

The ability of human genes to produce a number of different proteins helps explain the diversity among organisms that still have a relatively small percentage of variation in DNA. Among humans the similarity is measured to be around 99.5-99.8%. This leaves a small difference of only 0.2-0.5%. Yet this difference results in up to 15 million different nucleotides in humans (145).

4.2 Research in Alternative Splicing Detection

4.2.1 Automatic Methods for Detecting Alternative Splicing

Several research groups have used computational methods to analyze alternative splicing in the past decade. Some of them have studied ESTs through sequence alignment. Others have used different data sources, such as microarray data.

Automatic methods which use ESTs to detect splicing variants generally go through several steps. First, mRNA sequences are aligned to genomic contigs²⁰. Then, available ESTs are aligned to contigs. Next, sequences are clustered according to the genomic region where they are located. Finally, the alternative splices are defined as conflicting, contiguous, transitive or gapped. Most methods search for splice variants in a single organism. Other methods focus on detecting alternative splicing for a specific genome, such as the human or mouse genome. There are some methods that analyze splice variants by comparative analysis across species (140;142;146).

There are other forms of analysis which do not use EST sequences as a reference for identifying alternative splicing. Researchers can also search for a set of possible exons and splice sites among all exon concatenations for the highest homology to a protein domain. Other methods analyze microarray data to monitor splicing events based on known spliced variants (147). The tiling microarray uses probes²¹ that cover the entire genome or contigs of the genome in an unbiased fashion (148;149). One method uses Pfam²² information when clustering alternative splicing (150). Another process incorporates annotation integration resources (151).

4.2.2 Research of Automatic Applications in Alternative Splicing Detection

In the past decade, several papers have described different approaches for the automatic detection of alternative splicing. In 1999, Moronov used The Institute for

²⁰ A contig is a continuous sequence of DNA created by the assembly of overlapping fragments of a chromosome. The term also refers to a group of clones representing overlapping regions of a genome.

²¹ A probe is a single-stranded DNA molecule used in laboratory experiments to detect the presence of complementary sequence among a mixture of other single-stranded DNA molecules.

²² Pfam (<http://pfam.sanger.ac.uk>) is a large collection of multiple sequence alignments and hidden Markov models covering many common protein domains and families. For each Pfam family, users are able to check for multiple alignments, view protein domain architectures, examine species distribution, connect to other databases, and study known protein structures.

Genomic Research (TIGR²³) Human Gene Index as a reference for clustering the results after aligning ESTs to genomic DNA. The partial gene structures generated by spliced alignment were merged whenever they shared consecutive splicing sites spanning an intron. The superstructures corresponded to all possible gene structures for which each complete exon was supported by at least one of the alignments (142).

In 2000, Brett and his colleagues aligned ESTs to mRNA to identify insertions and deletions. Through analyzing the data, they identified alternative splicing genes (141). In the same year, Croft and his team created an intron database based on GenBank release 111. They identified ESTs that matched region previously only designated as intronic. With further examination, they found many unrecognized alternatively spliced exons, especially those in regions conserved between species (152).

In 2001, Kan and his colleagues aligned ESTs and genomic sequences to create transcripts for gene prediction. They elucidated gene structure as a complex problem that incorporated the splice junctions, gene boundaries, and alternative splicing. In addition, they analyzed shared regions of alternative splicing between the human and mouse genomes (146). In the same year, Modrek *et al.* aligned ESTs to genomic sequences using UniGene cluster information to detect alternative splices (137;153).

Lee and his group displayed alternative splicing graphically in 2003. Users can choose a cluster annotation and view splice variants within the same cluster (154). Johnson *et al.* further created a survey of exon junction microarrays based on known data (147).

²³ TIGR was a non-profit genomics research institute founded in 1992 by Craig Venter in Rockville, Maryland. It is now part of the J. Craig Venter Institute (<http://www.tigr.org>).

In 2004, Sorek's group distinguished alternative splices from constitutive exons (155). The next year, Hiller *et al.* used Pfam information to analyze alternative splicing events (150). Florea incorporated annotation integration resources for analysis in alternative splicing (151). Chen and his colleagues compared data across human, mouse and rat genomes the following year (140). Castrignano *et al.* established a database resource called ASPicDB for alternative splicing analysis in 2008. They updated their database based on NCBI Entrez gene and UniGene databases (156).

4.3 Applications for Alternative Splicing

Research in alternative splicing helps to identify genes and define the functional roles of genes. It also serves to analyze the complexity of alternative splices, particularly on the proteome. Moreover, it reveals the regulation of alternative splice forms across the whole genome and recognizes different terms of genes. Alternative splices can help to differentiate tissue specificities. Comparative genomics studies the evolution of alternative splicing. Recently, alternative splicing events have been studied in relation to diseases.

4.4 Case Study in Phosphodiesterase 4 (PDE4)

In order to determine how ESTs can be used to detect isoforms and tissue specificity with alternative splicing, a case study of PDE4 in the rat genome was conducted. This was a joint work with Mr. Christopher M. Whitaker from Dr. Nigel G. F. Cooper's laboratory in the Department of Anatomical Sciences and Neurobiology at the University of Louisville.

4.4.1 Phosphodiesterases (PDEs)

Phosphodiesterases (PDEs) are enzymes which act as regulators for signal transduction. Currently, there are 11 known families of PDEs. Each family includes three to four genes and each gene has three to eleven splice variants. PDEs degrade cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Some PDEs, such as PDE4, PDE7 and PDE8, degrade cAMP only. Some of them degrade cGMP only, including PDE5, PDE6, and PDE9. The rest of the PDEs – PDE1, PDE2, PDE3, PDE10, and PDE11 – degrade both cAMP and cGMP (Figure 4.4).

Figure 4.4: General scheme of cyclic 3', 5' nucleotide metabolism.

Image modified from Figure 1 in (157). Abbreviations are: cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; PDE1-PDE11, types (gene families) of PDE isozymes. The lines reflect the catalytic capacity of the pathway.

In general, all the PDE isoforms contain a catalytic site. PDEs are differentiated based on the N-terminus regions. The PDE4 family represents the largest PDE family, constituted by four genes (PDE4A, PDE4B, PDE4C and PDE4D). Moreover, PDE4 expresses in all tissues.

4.4.2 Motivation

Phosphodiesterase 4 (PDE4) is an enzyme that has been targeted for inflammatory disorders, including asthma and chronic obstructive pulmonary disease (COPD), as well as central nervous system (CNS) injury/disease. PDE4 has a large number of potential isoforms with apparent tissue and functional specificity. Potential roles of the different PDE4 isoforms within the CNS and other tissue types were studied using four known rat PDE4 paralogs (PDE4A, PDE4B, PDE4C and PDE4D) and their known isoforms. A computational method was designed to align rat ESTs to the corresponding chromosomal and mRNA sequences. The splice pattern and tissue type for each EST was examined in order to gain more insight into PDE4 isoform tissue specificity.

4.4.3 Phosphodiesterase 4 (PDE4)

PDE4, the most abundant PDE within the CNS (158), hydrolyzes cAMP (159) and selective inhibition by the drug rolipram (160). Recent studies have implicated PDE4 in many injury/disease models mostly through its role in inflammation (161) and have linked the enzyme to asthma, COPD and spinal cord injury (162-164), making it important for study. Some researchers have even shown that PDE4D is found in the blood of African American females who have had strokes (165).

In the human genome, the PDE4 family contains four known genes (PDE4A-D). Each gene has about three to eleven splice variants, or isoforms. Each isoform is defined by its unique N-terminus region. All have two to three conserved regions depending on the isoforms. These splice variants can be grouped into either long or short isoforms.

Currently, there are four PDE4 isoform classes in the human genome. They are long, short, super-short and dead-short isoforms (See Figure 4.5). Long isoforms have upstream conserved region 1 (UCR1) and 2 (UCR2) domains. Short isoforms do not contain UCR1. Super-short isoforms have an N-terminally truncated UCR2. The dead-short isoforms lack UCR1 and UCR2 and have an inactive catalytic unit that is both N- and C- terminally truncated.

Figure 4.5: PDE4 isoform diversity: long, short, super-short and dead-short.

Image adopted from Figure 2 in (166) and used by permission of author.

4.5 Methods for Computational Detection

A project for detecting PDE4 splice variants in the rat genome was designed and implemented *in silico* in order to discover how PDE4 is spliced and determine tissue specificity of the splice variants

4.5.1 Genomic Data

Curated mRNA sequences for the rat PDE4A, PDE4B, PDE4C and PDE4D genes and known isoforms were downloaded from NCBI/RefSeq (73-76). Release 18, dated 7-17-2006 (<http://www.ncbi.nlm.nih.gov/RefSeq/>). The size of the sequences ranged from 1,000 to 3,000 base pairs. GenBank ids for each of these sequences are provided in Table 4.1. Each of the mRNAs were positioned within the UCSC rn4 goldenpath assembly of the rat genome (81) using WU `blastn` (70;167) and BLAT (168).

PDE4A localized to chromosome 8; PDE4B to chromosome 5; PDE4C to chromosome 16; and PDE4D to chromosome 2 (Table 4.1). The corresponding repeat-masked genomic regions were downloaded from the UCSC rn4 goldenpath assembly (77-81) (<http://genome.ucsc.edu/>), dated 11-2004 including 30,000 bases flanking the 5' region of the first hit, and 30,000 bases flanking the 3' end of the final hit.

Table 4.1: Rat PDE4 Family mRNA Sequence Information

Gene	Variant	GenBank Accession	GenBank Identifier	Gene Length (bp)	Chromosome	Chromosomal Length (bp)
PDE4A	RefSeq	NM_013101.3	9681345	2,914	chr8	68,640
	1	L36467.1	703258	2,778		
	2	L27057.1	3334904	2,914		
	3	AF110461.2	12057232	2,315		
	4	L27062.1	436013	2,433		
	5	M26715.1	203982	2,202		
	6	M26716.1	203984	2,106		
	7	M26717.1	203986	2,071		
	8	M25348.1	206131	1,077		
	9	M28411.1	206053	1,077		
PDE4B	RefSeq	NM_017031.2	19923679	3,133	chr5	573,258
	1	AF202732.1	16930142	3,022		
	2	U95748.1	2352051	3,133		
	3	BC085704.1	55716044	2,511		
	4	AF202733.1	16930144	2,433		
	5	J04563.1	203967	2,158		
	6	L27058.1	950096	2,647		
	7	M25350.1	206126	1,080		
	8	M28413.1	206055	1,080		
PDE4C		XM_214325.4	19220990	2,140	chr16	23,713
	1	L27061.1	436011	1,764		
	2	M25347.1	206129	1,077		
PDE4D	RefSeq	NM_017032.1	8393931	2,077	chr2	*1,118,153
	1	AF536979.1	22901889	2,348		
	2	AF031373.1	2625099	2,470		
	3	EF102484.1	118500411	2,285		
	4	U09457.1	2564961	2,077		
	5	AY388961.1	37256003	2,157		
	6	EF121818.1	118772243	2,122		
	7	L27059.1	436007	2,142		
	8	L27060.1	3334454	2,834		
	9	U09455.1	517501	1,963		
	10	U09456.1	517416	1,821		
	11	AF536974.1	22901879	1,756		

Abbreviations: bp, base pair; chr, chromosome.

*Mapping of the rat PDE4D gene indicates sequence assembly errors within chromosome 2. The total region covered includes 1,114,915 bp of chromosome 2, 154 bp of chromosome 18 random fragments, and 3,084 bp of chromosome 2 random fragments.

4.5.2 Splice Detection

Expressed sequence tags (ESTs) were downloaded from GenBank/dbEST release 154.0, dated 6-15-2006 (<http://www.ncbi.nlm.nih.gov/dbEST/>) for the purpose of understanding how the sequences were spliced for each of the four PDE4 genes. PDE4 mRNA sequences were downloaded from NCBI/RefSeq release 17, dated 5-5-2006 (<http://www.ncbi.nlm.nih.gov/RefSeq/>). Each EST passed through five steps in the detection of splice patterns: data preprocessing, sequence comparison, fine tuning, data analysis and pattern recognition. Figure 4.6 illustrates this process.

Step 1: Data Preprocessing

EST sequences were parsed to include only those from the species *Rattus norvegicus*. Raw EST and PDE4 mRNA sequences were masked for repetitive elements using RepeatMasker (87;88). Low complexity regions for the resulting rat ESTs and mRNAs were then masked using the DUST (70), SEG (70;90) and XNU (70) filters available from the wuBLAST software (70;167).

Step 2: Sequence Comparison

The four PDE4 mRNA sequences were aligned to their corresponding genomic regions using sim4 (107), which determines the most likely intron/exon boundaries of the mRNAs by taking into account known donor/acceptor sites. An initial alignment of the ESTs to the genomic regions was performed using WU blastn.

Figure 4.6: Process flow chart in computational detecting section.

Step 3: Fine Tuning

EST sequences with less than 95% of percentage identity were filtered out, since these were not likely to be their true genomic locations. Remaining ESTs were realigned to the genome using sim4 (107), which returns the position where each EST appears in a chromosomal segment in accordance with known intron/exon boundary properties. The sim4 results were parsed to determine if the ESTs matched one contiguous region or if they spanned multiple regions, or exons. Each hit was therefore categorized as a single-exon hit or a multi-exon hit. Single-exon hits with a length less than 100bp were further filtered based on the number of hits in the region. If more than five ESTs hit the same region, all of ESTs for that region were discarded, since they most likely resulted from a previously uncharacterized repetitive element.

Step 4: Data Analysis

The ESTs remaining from Step 3 were divided into single-exon and multi-exon hit groups according to the gene to which they correspond: PDE4A, PDE4B, PDE4C or PDE4D. Each EST accession was searched against dbEST and UniGene (169). to determine the tissue type from which the EST was extracted, if known.

Step 5: Pattern Recognition

EST sequences that mapped to the same genomic location were placed in similar clusters. Overlapping patterns were examined for the maximum coverage length for each intron and exon. Each individual EST was examined for the pattern of exon and/or intron inclusion for the purpose of determining alternative splicing events, as well as possibly

determining previously undetected exonic regions. Single-exon hits were further condensed, since it was unknown if these were spurious, or actual hits to exonic regions.

4.6 Results in Exon Predictions

Predicted exon locations for each of the four known PDE4 classes were determined based on the sim4 (107) and BLAT (168) alignments of the RefSeq and alternatively spliced mRNA sequences to the genomic regions. PDE4A splits into thirty-three regions (exons) on chromosome 8 (Table 4.2), with a noticeable gap between patterns twelve and eighteen. Examination of the genomic assembly for this region indicates an unsequenced gap that should be closed with future sequencing and assembly. Regions of the RefSeq and nine known isoforms corresponding to this gapped region were multiply aligned using clustalW (170) to produce five aligned regions where a gap exists in the genome assembly. PDE4B maps to chromosome 5, and is split into 15 exons (Table 4.3) while PDE4C divided into 16 exons on chromosome 16 (Table 4.4). The results for the PDE4D class are more puzzling. One large 897 bp gap in the mRNA sequence exists after the predicted location for exon 11. There are 16.7 Mb that separates the predicted terminal exon from the rest of the structure (Table 4.5). In addition, the most likely hits for this 897 bp region are to unassembled fragments assigned to chromosome 18 of the rat genome. The most likely explanation for this missing region is incorrect assembly of the rat genome.

Table 4.2: PDE4A Exon Locations

Region Num.	CHROMOSOME 8	NM_013101.3 (2914 BP)	L36467.1 (2778 BP)	L27057.1 (2914 BP)	AF110461.2 (2315 BP)	L27062.1 (2433 BP)	M26715.1 (2202 BP)	M26716.1 (2106 BP)	M26717.1 (2071 BP)	M25348.1 (1077 BP)	M28411.1 (1077 BP)		14-1 Testes (F)	14-1 Testes (R)
1	CHR1 206,689,345 206,689,357								1-13					
2	CHR1 206,689,447 206,689,554								14-121					
3	20,176,941-20,176,964		7-30											
4	20,185,770-20,185,837		31-98											
5	20,189,322-20,189,507		99-284											
6	20,192,677-20,193,048	23-394		23-394										
7	20,210,557-20,210,642				1-86									
8	20,216,974-20,217,165	395-586	285-476	395-586	87-278									
9	20,218,045-20,218,081	587-623	477-513	587-623	279-315									
10	20,218,173-20,218,243	624-694	514-584	624-694	316-386									
11	20,221,145-20,221,194	695-744	585-634	695-744	387-436									
12	20,221,284-20,221,396	745-857	635-747	745-857	437-549									
13	xx,xxx,xxx-xx,xxx,xxx					1-266								
14	xx,xxx,xxx-xx,xxx,xxx					267-387	1-121							
15	xx,xxx,xxx-xx,xxx,xxx	858-901	748-791	858-901	550-593	388-431	122-165	26-69						
16	xx,xxx,xxx-xx,xxx,xxx	902-930	792-820	902-930	594-622	432-460	166-194	70-98	134-162					
17	xx,xxx,xxx-xx,xxx,xxx					461-480	195-214	99-118	163-182					
18	20,227,838-20,227,857	931-950	821-840	931-950	623-642									
	20,230,313-20,230,392												3-83	
19	20,230,483-20,230,572	951-1040	841-930	951-1040	643-732	481-570	215-304	119-208	183-272				84-172	150-240
20	20,230,573-20,230,680	1041-1148	931-1038	1041-1148	733-840	571-678	305-412	209-316	273-380	1-108	1-108		173-280	241-348
21	20,232,551-20,232,649	1149-1247	1039-1137	1149-1247	841-939	679-777	413-511	317-415		109-207	109-207		281-379	349-450
22	20,232,738-20,232,902	1248-1412	1138-1302	1248-1412	940-1104	778-942	512-676	416-580	381-545	208-372	208-372		380-544	451-615
23	20,234,075-20,234,124	1413-1512	1303-1352	1413-1512	1105-1154		677-726	581-630	546-595	373-422	373-422		545-595	616-665
24	20,234,125-20,234,174		1353-1402		1155-1204	943-1042	727-776	631-680	596-645	423-472	423-472		596-645	666-715
25	20,234,682-20,234,836	1513-1667	1402-1557	1513-1667	1205-1359	1043-1197	777-931	681-835	646-800	473-627	473-627		646-711... 20234745	716-769... 20,234,735
26	20,235,014-20,235,136	1668-1790	1558-1680	1668-1790	1360-1482	1198-1320	932-1054	836-958	801-923	628-750	628-750			
27	20,236,456-20,236,638	1791-1973	1681-1863	1791-1973	1483-1665	1321-1503	1055-1237	959-1141	924-1106	751-933	751-933			
28	20,240,619-20,240,762	1974-2117	1864-2007	1974-2117	1666-1809	1504-1647	1238-1381	1142-1285	1107-1250	934-1077	934-1077			
29	20,240,763-20,241,268	2118-2623	2008-2513	2118-2623	1810-2315	1648-2153	1382-1887	1286-1791	1251-1756					
30	20,241,269-20,241,527	2624-2882	2514-2772	2624-2882		2154-2411	1888-2148	1792-2052	1757-2017					
31	20,241,528-20,241,541	2883-2896		2883-2896		2412-2425	2149-2162	2053-2066	2018-2031					
32	20,241,542-20,241,557	2897-2912		2897-2912			2163-2178	2067-2082	2032-2047					
33	20,241,558-20,241,581						2179-2202	2083-2106	2048-2071					

Regions 8, 9, 10 (in red) correspond to the UCR1 region. Regions 12-17 (blue) correspond to the UCR2 region. Regions 20-29 (green) correspond to the catalytic region. The last two columns indicate the mapped sequence for 14-1 from testis tissue. Abbreviations: F, forward strand; R, reverse strand.

Table 4.3: PDE4B Exon Locations

Reg	CHR 5	NM_017031 3133 BP	AF202732 3022 BP	U95748 3133 BP	BC085704 2511 BP	AF202733 2433 BP	J04563 2158 BP	L27058 2647 BP	M25350 1080 BP	M28413 1080 BP	15-1 F Cereb.	15-1 F Cerv. Cord	23-1 F Brain	23-1 R Brain	23-1 F Cereb.	23-1 R Cereb.	23-1 F Retina	23-1 (R) Retina
1	122,960,641-122,960,883		10-253															
2	123,067,107-123,067,219		254-366															
3	123,079,695-123,079,933		367-605															
4	123,158,156-123,158,605	16-465		16-465														
5	123,158,606-123,158,887	466-747		466-747	1-282													
	123,168,982-123,168,984											1-3						
	123,168,985-123,169,154										1-172	4-174						
	123,169,166-123,169,172										173-179	175-181						
6	123,351,697-123,351,999					8-311												
7	123,416,343-123,416,537	748-942	606-800	748-942	283-477	312-506												
8	123,426,319-123,426,355	943-979	801-837	943-979	478-514	507-543												
9	123,426,452-123,426,465	980-988	838-846	980-988	515-523	544-552												
10	123,426,461-123,426,522	989-1050	847-908	989-1050	524-585	553-614	1-62											
11	123,434,704-123,434,753	1051-1100	909-958	1051-1100	586-635	615-664	63-112											
12	123,497,052-123,497,057							1-6										
13	123,497,150-123,497,802							7-659										
14	123,497,803-123,497,915	1101-1213	959-1071	1101-1213	636-748	665-777	113-225	680-772										
15	123,517,627-123,517,629																	2-4
	123,517,630-123,517,648													1-19		1-19		5-23
	123,517,649-123,517,656												8-15	20-27		20-27		24-31
	123,517,657-123,517,663												16-22	28-34	22-28	28-34		32-38
	123,517,664-123,517,899												23-259	35-270	29-264	35-272	25-260	39-275
16	123,518,627-123,518,720	1214-1307	1072-1165	1214-1307	749-842	778-871	226-319	773-866					260-354	271-365	265-358	273-369	261-354	276-370
17	123,523,794-123,523,861	1308-1375	1166-1233	1308-1375	843-910	872-939	320-387	867-934					355-422	366-433	359-426	370-437	355-422	371-438
18	123,523,862-123,523,972	1376-1486	1234-1344	1376-1486	911-1021	940-1050	388-498	935-1045	1-111	1-111			423-497	434-486	427-496	438-487	423-497	439-489
19	123,525,516-123,525,614	1487-1585	1345-1443	1487-1585	1022-1120	1051-1149	499-597	1046-1144	112-210	112-210								
20	123,525,751-123,525,915	1586-1750	1444-1608	1586-1750	1121-1285	1150-1314	598-762	1145-1309	211-375	211-375								
21	123,527,679-123,527,778	1751-1850	1609-1708	1751-1850	1286-1385	1315-1414	763-862	1310-1409	376-475	376-475								
22	123,529,535-123,529,689	1851-2005	1709-1863	1851-2005	1386-1540	1415-1569	863-1017	1410-1564	476-630	476-630								
23	123,529,781-123,529,903	2006-2128	1864-1986	2006-2128	1541-1663	1570-1692	1018-1140	1565-1687	631-753	631-753								
24	123,530,570-123,530,752	2129-2311	1987-2169	2129-2311	1664-1846	1693-1875	1141-1323	1688-1870	754-936	754-936								
25	123,533,054-123,533,197	2312-2455	2170-2313	2312-2455	1847-1990	1876-2019	1324-1467	1871-2014	937-1080	937-1080								
26	123,533,198-123,533,335	2456-2593	2314-2451	2456-2593	1991-2128	2020-2157	1468-1605	2015-2152										
27	123,533,336-123,533,612	2594-2870	2452-2728	2594-2870		2158-2433	1606-1882	2153-2429										
28	123,533,613-123,533,832	2871-3090	2729-2948	2871-3090			1882-2101	2430-2647										
29	123,533,833-123,533,877	3091-3133	2949-2993	3091-3133			2102-2146											
30	123,533,878-123,533,890		2994-3006				2146-2158											
31	123,533,891-123,533,899		3007-3015															

Regions 7-10 (in red) correspond to the UCR1 region. Regions 14-16 (blue) correspond to the UCR2 region. Regions 18-26 (green) correspond to the catalytic region. Abbreviations: F, forward strand; R: reverse strand.

Table 4.4: PDE4C Exon Locations

Region	CHR 16	XM_214325.4 2140 BP	L27061.1 1764 BP	M25347.1 1077 BP
1	19,220,990-19,220,909	1-82		
2	19,209,993-19,209,849	83-227		
3	19,202,151-19,201,990	228-389		
4	19,201,989-19,201,960	390-419	7-36	
5	19,201,551-19,201,515	420-456	37-73	
6	19,201,430-19,201,360	457-527	74-144	
7	19,201,236-19,201,184	528-580	145-197	
8	19,200,897-19,200,785	581-693	198-310	
9	19,200,708-19,200,615	694-787	311-404	
10	19,200,243-19,200,191	788-840	405-457	
11	19,200,190-19,200,083	841-948	458-565	1-108
12	19,199,997-19,199,899	949-1047	566-664	109-207
13	19,199,679-19,199,515	1048-1212	665-829	208-372
14	19,199,436-19,199,337	1213-1312	830-929	373-472
15	19,199,125-19,198,971	1313-1467	930-1084	473-627
16	19,198,573-19,198,451	1468-1590	1085-1207	628-750
17	19,198,259-19,198,077	1591-1773	1208-1390	751-933
18	19,197,643-19,197,500	1774-1917	1391-1534	934-1077
19	19,197,499-19,197,277	1918-2140	1535-1757	

Regions 3-6 (in red) correspond to the UCR1 region.
Regions 8-9 (blue) correspond to the UCR2 region.
Regions 11-19 (green) correspond to the catalytic region.

Table 4.5: PDE4D Exon Locations

Region	CHR 2	NM_017032.1 2077 BP	AF536979.1 2348 BP	AF031373.1 2470 BP	EF102484.1 2285 BP	U09457.1 2077 BP	AY388961 2157 BP	EF121818.1 2122 BP	L27059.1 2142 BP	L27060.1 2834 BP	U09455.1 1963 BP	U09456.1 1821 BP	AF536974.1 1756 BP
1	40,196,097-40,196,221		1-125										
2	40,394,274-40,394,503		126-355										
3	40,496,293-40,496,732			1-440									
4	40,644,734-40,645,025				1-292								
5	40,820,287-40,820,333	1-47				1-47							
6	41,010,800-41,010,928												
7	41,063,673-41,063,737						1-65	1-129					
8	41,114,938-41,115,127	48-239	356-547	441-632	293-484	48-239	66-257	130-321					
9	41,131,201-41,131,237	240-276	548-584	633-669	485-521	240-276	258-294	322-358					
10	41,136,439-41,136,449	277-287	585-595	670-680	522-532	277-287	295-305	359-369					
11	41,136,450-41,136,512	288-350	596-658	681-743	533-595	288-350	306-368	370-432	7-69				
12	41,139,162-41,139,211	351-400	659-708	744-793	596-645	351-400	269-418	433-482	70-119				
13	41,275,198-41,275,253										1-241		
14	41,275,254-41,275,397										242-285	1-144	
15	41,275,398-41,275,446										201-249		
16	41,275,447-41,275,483									13150	250-286		
17	41,275,484-41,275,596	401-513	709-821	794-906	646-758	401-513	419-531	483-595	120-232	37-150	287-399	145-257	
18	41,299,092-41,299,320												1-229
19	41,305,886-41,305,979	514-607	822-915	907-1000	759-852	514-607	532-625	596-689	233-326	151-244	400-493	258-351	230-323
20	41,307,257-41,307,429	608-780	916-1088	1001-1173	853-1025	608-780	626-798	690-862	327-499	245-417	494-666	352-524	324-496
21	41,308,372-41,308,470	781-879	1089-1187	1174-1272	1026-1124	781-879	799-897	863-961	500-598	418-516	667-765	525-623	497-595
22	41,309,604-41,309,768	880-1044	1188-1352	1273-1437	1125-1289	880-1044	898-1062	962-1126	599-763	517-681	766-930	624-788	596-760
23	41,310,913-41,311,012	1045-1144	1353-1452	1438-1537	1290-1389	1045-1144	1063-1162	1127-1226	764-863	682-781	931-1030	789-888	761-860
	CHR 18 RANDOM												
24	266,263-266,417	1145-1299	1453-1607	1538-1692	1390-1544	1145-1299	1163-1317	1227-1381	864-1018	782-936	1031-1185	889-1043	861-1015
	CHR 2 RANDOM												
25	1,582,343-1,582,465	1300-1422	1608-1730	1693-1815	1545-1667	1300-1422	1318-1440	1382-1504	1019-1141	937-1059	1186-1308	1044-1166	1016-1138
26	1,583,164-1,583,346	1423-1605	1731-1913	1816-1998	1668-1850	1423-1605	1441-1623	1505-1687	1142-1324	1060-1242	1309-1491	1167-1349	1139-1321
27	1,583,844-1,584,278	1606-2040	1914-2348	1999-2433	1851-2285	1606-2040	1624-2058	1688-2122	1325-1759	1243-1677	1492-1925	1350-1784	1322-1756
28	1,584,279-1,584,308	2041-2070		2434-2463		2041-2070	2059-2088			1760-1789	1678-1707	1926-1955	1785-1814
29	1,584,309-1,584,377						2089-2157			1790-1858	1708-1776		
30	1,584,378-1,584,630								1859-2111	1777-2029			
31	1,584,631-1,584,638	2071-2077		2464-2470		2071-2077			2112-2119	2030-2037	1956-1963	1815-1821	
32	1,584,639-1,584,644								2120-2126	2038-2043			
33	1,584,645-1,585,427									2044-2827			

4.6.1 Spliced Pattern Observation

A total of 812,497 rat ESTs were extracted from dbEST and compared to the genomic regions for the PDE4 families (See Section 4.5). ESTs directly correlate to processed mRNA sequences expressed in the cell, and therefore yield information pertaining to the spliced exon patterns. While EST sequences contain information about adjacent exons alternatively spliced, they normally do not contain the whole isoform due to their short length (normally 200-500 bp). A breakdown of the tissue types in which ESTs were found for each of the four PDE4 families is given in Figure 4.7.

Figure 4.7: Tissue distribution of ESTs for each of the four PDE4 families.
Unknown, mixed and uncharacterized tissue types have been removed.

Variant tissue types were found in each PDE4 gene, when tracing the information from the GenBank data. Brain, genitourinary, heart and pancreas tissues were found in all PDE4 genes. Adipose and embryonic tissues were only associated with PDE4A; endocrine and vestibular end organs tissues were found specifically for PDE4B; the tissue types of cervix, dorsal root ganglia, hippocampus, hypothalamus and insulinoma were found with only PDE4C; and the cartilage tissue was only associated with PDE4D (Figure 4.7).

4.6.2 PDE4A

An initial filtering of EST hits with a percentage identity of 95% or greater yields 742 ESTs for the PDE4A region. After additional filtering and clustering, 27 ESTs were shown to map uniquely to the PDE4A region of chromosome 8. Eighteen of these were found to be unspliced ESTs, involving four different single-exon patterns. Another nine were spliced ESTs, representing nine separate multiple exon patterns (Tables 4.6 and 4.7). The ESTs for PDE4A were found in following tissues: four each for the brain and genitourinary system and one each for heart, adipose, embryonic, placenta, pineal gland, testis, pancreas, kidney and eye with the remainder coming from unknown, uncharacterized or mixed tissue.

Twelve of the single exon hits represented possible extensions of the 3' untranslated region (UTR) of known PDE4A isoforms. Another EST hit, from accession BG372498.1 (pattern 1), indicated a potential new exon between regions six and seven from Table 4.2. This EST was derived from eye tissue, and therefore might have some tissue specificity.

Table 4.6: Single Exon Splicing Patterns and Tissue Types for PDE4A

Pattern	Accession	Tissue Type	Regions Covered	Notes	Chromosomal	locations
1	BG372498.1	eye	(6-7)	new exon	20,197,794	20,198,264
2	CB797062.1	adipose	28-29		20,240,617	20,240,944
2	CA506060.1	embryonic	29		20,240,875	20,241,552
2	CR458483.1	uncharacterized	29-30-31		20,241,248	20,241,539
2	BF413211.1	brain	29-30-31-32		20,241,264	20,241,552
3	BG378996.1	brain	30-31-32-33		20,241,302	20,241,574
3	CV111386.1	genitourinary	30-3132-33-(33-)	3' UTR extension	20,241,477	20,242,162
4	AW507088.1	brain	(33-)	3' UTR extension	20,241,883	20,242,178
4	BF545214.1	mixed	(33-)	3' UTR extension	20,242,103	20,242,242
4	CR459446.1	uncharacterized	(33-)	3' UTR extension	20,242,379	20,242,721
4	AI029179.1	mixed	(33-)	3' UTR extension	20,242,531	20,242,727
4	AA999059.1	unknown	(33-)	3' UTR extension	20,242,535	20,242,721
4	BE120273.1	mixed	(33-)	3' UTR extension	20,242,546	20,242,723
4	BG381367.1	heart	(33-)	3' UTR extension	20,242,547	20,242,727
4	BQ192530.1	uncharacterized	(33-)	3' UTR extension	20,242,556	20,243,158
4	BF399702.1	mixed	(33-)	3' UTR extension	20,242,560	20,242,721
4	BE107614.1	mixed	(33-)	3' UTR extension	20,242,697	20,243,128
4	CR465548.1	uncharacterized	(33-)	3' UTR extension	20,242,792	20,243,158

Five of the spliced EST patterns revealed potential new exons (Table 4.7). These occurred between regions two and three (pattern 10 from genitourinary tissue); between regions six and seven (pattern 13 from kidney tissue); between regions 18 and 19 (pattern 14 from genitourinary tissue); between regions 22 and 23 (pattern 8 from pancreas tissue); and between regions 24 and 25 (pattern 6a derived from whole brain tissue). The first three splice patterns (patterns 1, 10 and 13) occurred upstream of the UCR1 in the hypervariable N-terminal region. Pattern 14 produces a novel exon in the linker region between UCR2 and the conserved catalytic core while patterns 8 and 6a produced novel exons in the catalytical core of PDE4A. In addition, a new splicing pattern incorporating known exons was described by pattern 10, derived from genitourinary tissue.

Table 4.7: Multiple Exon Splicing Patterns and Tissue Types for PDE4A

Pattern	Accession	Tissue Type	Regions Covered	Notes	Chromosomal	locations
5	CB770386.1	peneal gland	27-28-29		20,236,527	20,236,638
					20,240,619	20,240,896
6	CB702066.1	placenta	18-19		20,227,838	20,227,858
					20,230,484	20,230,531
6a	DY312415.1	whole brain	18-19-20-21-22-23-24-(24-25)	new exon	20,227,838	20,227,858
					20,230,484	20,230,680
					20,232,551	20,232,649
					20,232,738	20,232,902
					20,234,075	20,234,170
7	CV121499.1	genitourinary	19-20-21-22-23-24-25-26		20,234,614	20,234,623
					20,230,499	20,230,680
					20,232,551	20,232,649
					20,232,738	20,232,902
					20,234,075	20,234,174
8	BP485815.1	pancreas	(22-23)-23-24-25-26-27	new exon	20,234,682	20,234,836
					20,235,014	20,235,136
					20,235,014	20,235,127
					20,236,456	20,236,569
10	CV120906.1	genitourinary	(2-3)-3-5-8-9-10-11-12	new exon; new splice pattern	20,233,889	20,234,174
					20,234,682	20,234,836
					20,235,014	20,235,136
					20,236,456	20,236,569
13	CK482793.1	kidney	(6-7)-7-8	new exon	20,176,887	20,176,964
					20,189,322	20,189,507
					20,216,974	20,217,165
					20,218,045	20,218,081
14	CK469415.1	genitourinary	(18-19)-19-20-21-22	new exon	20,218,173	20,218,243
					20,221,145	20,221,193
					20,221,284	20,221,394
					20,210,157	20,210,642
15	CK652857.1	testis	21-22-23-24-25-26-27		20,216,974	20,217,154
					20,229,884	20,229,889
					20,230,041	20,230,392
					20,230,484	20,230,680
					20,232,551	20,232,649
					20,232,738	20,232,868
					20,232,640	20,232,649
					20,232,738	20,232,902
					20,234,075	20,234,174
					20,234,682	20,234,836
					20,235,014	20,235,136
					20,236,456	20,236,638

Figure 4.8: PDE4A isoforms, EST patterns and sequenced regions.

Abbreviations: F, forward strand; R, reverse strand.

4.6.3 PDE4B

Initial filtering resulted in 5,112 ESTs which matched the PDE4B genomic region. After clustering and filtering again, 49 ESTs were found to map completely and uniquely to the PDE4B region of the rat chromosome 5. Thirty-eight of these ESTs were from single-exon hits covering 14 different regions of PDE4B while 11 were from multiple-exon hits representing seven different regions (Tables 4.8 and 4.9). Fifteen of the ESTs originated from brain, seven from heart, three from pancreas, two each from endocrine and genitourinary, and one each from vestibular end organs, lung, and placenta. The remaining were mixed, uncharacterized or unknown.

Eighteen of the single-exon hits indicated extensions of the 3' UTR region (pattern 10). Another 11 provide evidence for an exon between regions five and six (patterns 1, 2, 3, 4, 5, 6 and 17). Pattern 16 indicated a new exon between regions six and seven, while pattern 22 indicated a new exon between regions 11 and 12. In addition, two supported a previously discovered exon between regions 14 and 15 (patterns 7 and 8), and another indicated a novel exon between regions 20 and 21 (pattern 9).

Three of the spliced EST patterns indicated previously discovered exons. These include two exons between regions five and six (pattern 15), one exon between regions 11 and 12 (pattern 24), one exon between regions 14 and 15 (pattern 23), two exons between regions 15 and 16 (pattern 23), and one novel exon between regions 16 and 17 (pattern 23).

Of the novel predicted exons, a number were in the N-terminal domain (patterns 1, 2, 3, 4, 5, 6, 15, 16 and 17) while two were in the linker region between UCR1 and UCR2 (patterns 22 and 24), five were in the UCR2 region (patterns 7, 8 and three from

Table 4.8: Single Exon Splicing Patterns and Tissue Types for PDE4B

Pattern	Accession	Tissue Type	Regions Covered	Notes	Chromosomal	locations
13	BF542776.1	mixed	4-5		123,158,439	123,158,759
13	AI535520.1	mixed	4-5		123,158,459	123,158,763
1	BP471352.1	pancreas	(5-6)	new exon	123,168,205	123,168,508
1	BP471550	pancreas	(5-6)	new exon	123,168,205	123,168,703
17	BF386412.1	mixed	(5-6)	new exon	123,169,509	123,169,999
2	BE107554	mixed	(5-6)	new exon	123,229,629	123,230,075
3	BF566271	mixed	(5-6)	new exon	123,246,032	123,246,713
3	BF412632	brain	(5-6)	new exon	123,246,202	123,246,736
3	BF390665	brain	(5-6)	new exon	123,246,238	123,246,736
3	AW527944	brain	(5-6)	new exon	123,246,283	123,246,735
4	BF565113	brain	(5-6)	new exon	123,318,158	123,318,617
5	AW528686	brain	(5-6)	new exon	123,318,771	123,319,133
6	BE120216	mixed	(5-6)	new exon	123,324,159	123,324,766
16	BF390202.1	mixed	(6-7)	new exon	123,367,916	123,368,339
22	BF390420.1	mixed	(11-12)	new exon	123,493,547	123,493,870
7	DV713714	vestibular end organs	14-(14-15)	new exon	123,497,867	123,498,457
8	CB806131	endocrine	(14-15)	new exon	123,503,851	123,504,248
9	CB738137	brain	(20-21)	new exon	123,526,313	123,526,716
10	BE097208	uncharacterized	27-28-29-30-31-(31-)	3' UTR extension	123,533,385	123,533,907
10	AI102296	genitourinary	27-28-29-30		123,533,406	123,533,890
10	CR469935	genitourinary	27-28		123,533,494	123,533,829
10	CO399629	brain	27-28-29-30-31-(31-)	3' UTR extension	123,533,552	123,534,298
10	CB792692	whole brain	28-29-30-31-(31-)	3' UTR extension	123,533,775	123,534,202
10	AW141098	heart	28-29-30-31-(31-)	3' UTR extension	123,533,819	123,534,392
10	CB766615	brain	28-29-30-31-(31-)	3' UTR extension	123,533,823	123,534,193
10	CO405696	brain	29-30-31-(31-)	3' UTR extension	123,533,876	123,534,550
10	CB765975	lung	31-(31-)	3' UTR extension	123,533,894	123,534,258
10	CF110282	heart	(31-)	3' UTR extension	123,534,213	123,534,898
10	AI104604	heart	(31-)	3' UTR extension	123,534,853	123,535,238
10	AI104055	heart	(31-)	3' UTR extension	123,534,858	123,535,314
10	AI104808	mixed	(31-)	3' UTR extension	123,534,927	123,535,330
10	BF559266	whole brain	(31-)	3' UTR extension	123,534,510	123,534,951
10	AA858930	mixed	(31-)	3' UTR extension	123,534,832	123,535,333
10	CB720726	mixed	(31-)	3' UTR extension	123,534,859	123,535,333
10	BM387577	mixed	(31-)	3' UTR extension	123,534,880	123,535,333
10	BE120980	mixed	(31-)	3' UTR extension	123,534,895	123,535,333
10	BE119478	mixed	(31-)	3' UTR extension	123,534,902	123,535,324
10	BE119775	uncharacterized	(31-)	3' UTR extension	123,534,912	123,535,333

Table 4.9: Multiple Exon Splicing Patterns and Tissue Types for PDE4B

Pattern	Accession	Tissue Type	Regions Covered	Notes	Chromosomal	locations
11	CB807697.1	brain	20-21-22		123,525,756	123,525,915
					123,527,679	123,527,778
					123,529,535	123,529,598
11	CB773843.1	endocrine	20-21-22-23		123,525,822	123,525,915
					123,527,679	123,527,778
					123,529,535	123,529,689
					123,529,781	123,529,815
12	CB784576.1	mixed	5-7-8		123,158,708	123,158,887
					123,416,343	123,416,537
					123,426,319	123,426,338
12	CK599899.1	heart	5-7-8-9-10-11		123,158,608	123,158,887
					123,416,343	123,416,537
					123,426,319	123,426,355
					123,426,452	123,426,522
					123,434,704	123,434,753
15	BP483665.1	pancreas	(5-6)-(5-6)	new exons	123,168,478	123,169,118
					123,169,135	123,169,249
19	CK599213..1	heart	13-14-15-16-17-18		123,497,581	123,497,915
					123,518,627	123,518,720
					123,523,794	123,523,973
19	CV109674.1	placenta	13-14-15-16-17-18-19-20		123,497,681	123,497,915
					123,518,627	123,518,720
					123,523,794	123,523,972
					123,525,516	123,525,615
					123,525,753	123,525,814
20	AA799729.1	heart	13-14-(31-)	new splice	123,497,541	123,497,915
					123,535,118	123,535,311
24	CB718665.1	brain	(11-12)-14-16-17-18-19		123,496,114	123,496,167
					123,497,803	123,497,915
					123,518,627	123,518,720
					123,523,794	123,523,972
					123,525,516	123,525,596
23	BF563905.1	brain	(14-15)-15-(15-16)-16	new exons	123,517,507	123,517,899
					123,518,627	123,518,710
23	AW531150.1	brain	(15-16)-16-(16-17)-17	new exons	123,517,692	123,517,899
					123,518,627	123,518,836

pattern 23), one in the linker region between UCR2 and the catalytic core (pattern 23), and one was inside the catalytic core itself (pattern 23). In addition, a novel splicing pattern of known exons was found in pattern 20 derived from heart EST tissue.

Figure 4.9: PDE4B isoforms, multiple EST patterns and sequence hits.
 Abbreviations: F, forward strand; R, reverse strand.

4.6.4 PDE4C

A total of 50 ESTs initially mapped to the PDE4C region. Clustering and filtering reduced this number to twelve ESTs, four representing single-exon hits and eight representing spliced exons (Table 4.10). Four of these ESTs originate from pancreas tissue, two from insulinoma, and one each from kidney, lung and placenta. The remaining ESTs came from mixed or unknown tissue types.

A total of three separate single-exon patterns were detected. Each represented a potential novel exon between regions one and two. Seven separate multiple exon patterns appeared. Up to eleven previously uncharacterized exons between regions one and two exist, as indicated by patterns 2, 23, 24, 26, 27 and 28. In addition, there appear to be two new exons between patterns four and five (pattern 1), one between regions five and six, and one between regions six and seven (pattern 1). The new exons predicted between regions one and two occur in the N-terminal domain, while the exons predicted between regions four and five, and between five and six occur in UCR1. The exon predicted between regions six and seven occur in the linker region between UCR2 and the conserved catalytic core. The detection of a large number of potentially uncharacterized exons in the N-terminal domain provides great insight since only three isoforms were previously described for the rat PDE4C family of genes.

Table 4.10: Splicing Patterns and Tissue Types for PDE4C

	Pattern	Accession	Tissue Type	Regions Covered	Notes	Chromosomal	locations
single exon	3	AW921872.1	mix	(1-2)	new exon	19,216,126	19,216,545
	4	BP498358.1	insulinoma	(1-2)	new exon	19,217,290	19,217,705
	4	BP502440.1	insulinoma	(1-2)	new exon	19,217,292	19,217,839
	25	BP484004.1	pancreas	(1-2)	new exon	19,218,256	19,218,972
multi exon	1	BP471523.1	pancreas	(6-7)-6-(5-6)-5-(4-5)-(4-5)	new exons	19,201,287	19,201,766
						19,201,883	19,201,887
	1	BP478640.1	pancreas	(6-7)-6-(5-6)-5-(4-5)-(4-5)	new exons	19,201,335	19,201,766
						19,201,883	19,201,887
	2	AI233747.1	kidney	(1-2)-(1-2)	new exons	19,214,941	19,215,074
						19,215,431	19,215,680
	23	CF114754.1	lung	(1-2)-(1-2)	new exons	19,217,002	19,217,281
						19,217,832	19,218,050
	24	BM389421.1	mix	(1-2)-(1-2)	new exons	19,217,276	19,217,788
						19,217,987	19,218,032
	26	BP474083.1	pancreas	(1-2)-(1-2)	new exons	19,218,709	19,218,990
						19,219,043	19,219,241
	27	CV126227.1	placenta	(1-2)-(1-2)-1-(1-)	new exons	19,218,107	19,218,147
						19,218,828	19,219,238
						19,220,903	19,221,016
	28	BQ200281.1	unknown	(1-2)-(1-2)-(1-2)-1	new exons	19,217,317	19,217,783
						19,219,088	19,219,196
						19,219,201	19,219,238
						19,220,903	19,220,985

Figure 4.10: PDE4C isoforms, multiple EST patterns and sequence hits.

4.6.5 PDE4D

PDE4D had an inordinately large number of EST hits (38,742). However, most of these fell into a single exonic region that appeared to be a previously uncharacterized rat repetitive element. Once these hits are removed, 18 single-exon and three multiple-exon hits remain (Table 4.11).

The 18 single-exon hits produced 15 unique patterns, each of which suggested novel exons. Up to seven novel exons were detected between regions five and six (patterns 1, 2, 3, 4, 5 and 22) with an additional five between regions six and seven (patterns 6, 7, 8 and 9), three between regions 12 and 13 (patterns 11, 12 and 13), and one each between regions eight and nine (pattern 10) and between regions 17 and 18 (pattern 14).

The three spliced EST hits create three separate patterns. Pattern 21 suggested two new exons, one between regions six and seven, and one between regions seven and eight. Pattern 16 suggested a single novel exon between regions 12 and 13, while pattern 17 revealed an exon between regions 20 and 21.

Fourteen of the predicted exons occurred in the 5' N-terminal domain (as indicated from patterns 1, 2, 3, 4, 5, 6, 7, 8, 9, 21 and 22), one was in the UCR1 region (pattern 10), five were in the linker region between UCR1 and UCR2 (patterns 11, 12, 13 and 16), one was in the UCR2 region (pattern 14), and one is in the catalytic core (pattern 17).

Table 4.11: Splicing Patterns and Tissue Types for PDE4D

	Pattern	Accession	Tissue Type	Regions Covered	Notes	Chromosomal	locations
single exon	1	CF106982.1	lung	(5-6)	new exon	40,821,601	40,822,129
	2	CB735250.1	brain	(5-6)	new exon	40,844,702	40,845,000
	3	BF561343.1	mixed	(5-6)	new exon	40,928,435	40,928,658
	3	AA99742.1	mixed	(5-6)	new exon	40,928,541	40,929,191
	4	CR471157.1	uncharacterized	(5-6)	new exon	40,938,872	40,939,138
	22	CF114850.1	lung	(5-6)	new exon	40,962,055	40,962,763
	5	CR460313.1	uncharacterized	(5-6)	new exon	40,988,404	40,988,701
	6	BF567734.1	brain	(6-7)	new exon	41,018,068	41,018,402
	6	AW522698.1	brain	(6-7)	new exon	41,018,068	41,018,471
	7	CF107424.1	lung	(6-7)	new exon	41,055,789	41,056,469
	8	BG379252.1	brain	(6-7)	new exon	41,060,267	41,060,631
	9	AW434715.1	heart	(6-7)	new exon	41,063,099	41,063,498
	10	BF401594.1	mixed	(8-9)	new exon	41,119,841	41,120,456
	11	BQ192875.1	uncharacterized	(12-13)	new exon	41,145,546	41,145,899
multi exon	12	CF113676.1	lung	(12-13)	new exon	41,272,381	41,272,835
	13	BG377764.1	genitourinary	(12-13)	new exon	41,274,996	41,275,383
	13	BE118751.1	mixed	(12-13)	new exon	41,274,997	41,275,392
	14	BE103213.1	brain	(17-18)	new exon	41,284,770	41,285,306
	16	BP469236.1	pancreas	11-12-(12-13)-17-19-20	new exon	41,136,455	41,136,512
						41,139,162	41,139,211
						41,153,803	41,153,963
						41,275,484	41,275,548
						41,275,555	41,275,596
						41,305,886	41,305,979
	17	BF566977.1	heart	19-20-(20-21)	new exon	41,305,945	41,305,979
						41,307,257	41,307,451
	21	CB322599.1	cartilage	(6-7)-7-(7-8)	new exon	41,063,589	41,063,737
						41,065,963	41,066,486

Figure 4.11: PDE4D isoforms, multiple EST patterns and sequence hits (chromosome 2).

Figure 4.12: PDE4D isoforms, multiple EST patterns and sequence hits (chromosome 2 RANDOM).

4.7 Wet Lab Methods

Select sequence patterns found by computational methods were further tested for verification in the wet lab using reverse transcriptase polymerase chain reaction (RT-PCR) and DNA sequencing.

4.7.1 Polymerase Chain Reaction (PCR)

PCR, or polymerase chain reaction, amplifies specific DNA segments by exploiting certain features of DNA replication *in vitro*²⁴. The method was developed by the Cetus²⁵ scientist Kary Mullis in 1983 (171;172). The PCR technique won Mullis a shared Nobel Prize in Chemistry “for his invention of the polymerase chain reaction

²⁴ *In vitro* is a test performing in glass or plastic vessels in the laboratory.

²⁵ Cetus Corporation was one of the first biotechnology companies. It was established in Berkeley, California in 1971. Cetus Corp. developed several significant pharmaceutical drugs as well as a revolutionary DNA amplification technique. Cetus Corp. was merged with Chiron Corporation in 1991 and Chiron Corp. was further integrated into Novartis Pharmaceuticals (<http://www.novartis.com/>) in 2006.

(PCR) method,” along with Michael Smith “for contribution to the developments of methods within DNA-based chemistry.”

The PCR technique uses a pair of primers, which are DNA segments approximately 20 bp long that complement to the DNA being synthesized and a thermo stable DNA polymerase to achieve near-exponential enzymatic amplification of target DNA. PCR proceeds in three stages: denature double-stranded DNA, anneal primer, and extend the annealed primers (171-174). Figure 4.13 shows the basic PCR cycle.

Figure 4.13: Basic steps in the PCR cycle.

Hatched lines indicate target DNA. Filled boxes represent annealed primers.

4.7.2 RT-PCR

RT-PCR, abbreviated for reverse transcriptase polymerase chain reaction, is a highly sensitive technique for the detection and quantization of mRNA. This technique includes two parts: the synthesis of cDNA from RNA by reverse transcript and the amplification of a specific cDNA by the polymerase chain reaction.

4.7.3 DNA Sequencing

Sequences which had been amplified by the RT-PCR method were extracted and run through DNA sequencing. If the target sequences found from computation matched the sequences from the wet lab, it would highly to be verified the presence of novel exons and which had not been reported.

4.7.4 Splice Exon Verification

The novel exons predicted from computation with a length of at least 25 bases and a sequence similarity of at least 98% to the genomic regions were studied using RT-PCR and DNA sequencing techniques. PCR primer pairs were detected using MPrime 1.3 (175). Since each of the predicted exons were located upstream of the catalytic core which should be expressed in all viable variants, one primer was localized within the predicted exon and the other anchored inside of the catalytic core. A primer min, max, and opt size of 20 bp was incorporated with a primer GC%²⁶ min of 30%, max of 60% and opt of 50%. The melting temperature was set to a minimum of 57, max of 63, and optimum of 60. A product minimum size of 395 bases was used with a maximum of 405 and an optimum of 400, except for those cases where one primer could not be found anchored in the predicted exon. For those cases, a min value of 195 was used with a max of 205 and an opt of 200. A GC clamp of 2 was incorporated.

²⁶ GC% represents the percentage of total number of G's and C's in the sequence. For instance, GC% 30% means that the number of G's and C's occur 30% in the sequence

4.8 Results in Verified Predicted Exons

4.8.1 Tissue Types

The tissue types were found for the splice patterns in each PDE4 gene in computational methods were sequenced using RT-PCR. Tissues from rat liver, lung, sciatic nerve, testes, heart, brain (cortex), cerebellum, spinal cord, skeletal muscle, spleen, optic nerve, kidney, small intestine, retina, aorta and seminal vesicle were extracted and verified.

4.8.2 Predicted Exons Verification

In order to validate the proposed computational approach as an accurate predictor of novel isoforms, three novel splice patterns showing the potential for tissue specificity were chosen for further study. These included pattern 14 from PDE4A, patterns 15 and 23 from PDE4B, and patterns 21 and 22 from PDE4D. PCR primers were designed using MPrime (175). (See Section 4.7.4). All primers (Shown in Table 4.12) were then used to perform RT-PCR on RNA extracted from rat liver, lung, sciatic nerve, testes, heart, brain (cortex), cerebellum, spinal cord, skeletal muscle, spleen, optic nerve, kidney, small intestine, retina, aorta and seminal vesicle.

Table 4.12: PCR Primer Pairs for Selected Predicted Exons

Gene Family	Pattern	Forward Primer	Reverse Primer
PDE4A	14-1	CCAGAACCATCCCTAAGACC	TGTCGCAGTTCTCTTCTTGC
PDE4B	15-1	GTGTGCTCTGGAATTTTGG	TACATGATGCATGTGAGGGG
PDE4B	23-1	ATAAGCAGGGAGCCCATAGG	GACTCCAAAGCGTGAGATGC
PDE4D	21-1	CACAACAACATCCATGCTGC	TCACCTTCTTCGTTTCAACC
PDE4D	21-2	CTCCTTATGCCACTGACACG	CACAGTTTTCTTCCTGGAGC
PDE4D	22-1	TCAGTCCAAGAAGATTGCCG	CAGCCAGCAGATTCATGTGC
PDE4D	22-2	CACAACAACATCCATGCTGC	TCACCTTCTTCGTTTCAACC

Reverse transcriptase PCR results indicate that PDE4A pattern 14 is tissue specific to testes with a single band occurring at approximately 900 bp (Figure 4.14). PDE4B pattern 23 is expressed in brain cortex, cerebellum and retina with a strong band between 500 and 600 bp and additional two lighter bands appearing for brain and cerebellum (Figure 4.15, lower panel). PDE4B pattern 15 appears for cerebellum and spinal cord at approximately 300 bp (Figure 4.15, upper panel). PDE4D patterns 21 and 22 show very strong banding patterns at 400 bp for every tissue type, with the exception of seminal vesicle for pattern 22 (Figure 4.16). The results for the PDE4D patterns were expected since the PCR primers were designed within the conserved catalytic core.

Figure 4.14: RT-PCR for PDE4A pattern 14.

Figure 4.15: RT-PCR for PDE4B patterns 15 and 23.

Figure 4.16: RT-PCR for PDE4D patterns 21 and 22.

Next, DNA sequencing was used to confirm that the observed bands corresponded to the gene region of interest using the forward and reverse PCR primers as independent sequencing primers (See Section 4.7.4). The resulting sequences are shown in Tables 4.13-4.15.

For PDE4A pattern 14, the band found in testes corresponded to the PDE4A region of chromosome 8. Mapping of the sequence is shown in Table 4.2 and Figure 4.3. The sequencing confirmed additional exons for PDE4A pattern 14 that were not found in any known isoforms.

Two patterns were considered for PDE4B. The resulting sequence maps are provided in Table 4.3 and Figure 4.4. For PDE4B pattern 15, two bands were extracted and sequenced: the cerebellum and spinal cord bands occurring at 300 bp. Pattern 23 had three bands extracted and sequenced, for the brain, cerebellum, and retina. Sequencing confirmed that all of these bands were unique to PDE4B and revealed new exons previously uncharacterized for PDE4B isoforms.

The strong banding patterns for patterns 21 and 22 of PDE4D were extracted and sequenced as well (results not shown). The resulting sequences map uniquely back onto the catalytic region for PDE4D.

Table 4.13: Sequencing Results (Primer 14-1 in PDE4A)

```
>14-1_PDE4A_TestesForwardPrimer
AGGAAAAGTACTTACATGTGTCCCATGTTTCAAACCTCAGAGAAGAGACCAGCATTGCCGTC
CGCAGGATGACCTCATATCAAACAAGCAGAATGAAGTGGAGATCCCCTCACCCACACCTCGG
CAGAGAGCCTTCCAGCAGCCCCCGCCGTCAGTGCTGCGACAGTCCCAGCCCATGTCTCAGAT
CACAGGGCTGAAAAAGCTGGTACACACTGGAAGCTTGAACACCAACGTCCCACGGTTTGGAG
TCAAGACAGATCAAGAGGACCTCTTAGCACAAGAAGTGGAGAACTTGAGCAAATGGGGCCTG
AACATCTTTTGTGTGTGTCGGAGTACGCTGGAGGCCGCTCACTCAGCTGTATCATGTATACAAT
ATTCCAGGAGCGGGACCTACTGAAGAAATCCACATCCCTGTGGACACCATGATGATGTACA
TGCTGACCCTGGAGGACCACTACCATGCCGACGTGGCCTACCACAACAGCCTGCACGCAGCG
GATGTGCTGCAGTCCACACACGTGCTGCTGGCCACGCCCGCACTGGACGCTGTGTTCTACTG
ACCTGGAGATTCTTGCTGCCCTCTTCGCTGCTGCCATCCACGATGTGGACCACCCTGGCGTC
TCCACCCAGTTCCTAATCAACACCAATTCGGAGCTGGCGTTGATGTACAACAATGAGTCTGT
GCTTGGAACCAACACCTGGGCTGTGGGGATCCAAGCCGCCCCAAAAAACAACCTGCAA
AACANGAANACTCNGTGGNGGNN

>14-1_PDE4A_TestesReversePrimer
GGTGGTGGTTCTCAAGCACAGACTCATCGTTGTACATCAACGCCAGCTCCGAATTGGTGTTG
ATTAGGAACTGGTTGGAGACGCCAGGGTGGTCCACATCGTGGATGGCAGCAGCGAAGAGGGC
AGCAAGAATCTCCAGGTCTGTGAACACAGCGTCCAGTGCGGGCGTGGCCAGCAGCACGTGTG
TGGACTGCAGCACATCCGCTGCGTGCAGGCTGTTGTGGTAGGCCACGTCGGCATGGTAGTGG
TCCTCCAGGGTCAGCATGTACATCATCATGGTGTCCACAGGGATGTGGAATTTCTTCAGTAG
GTCCCGCTCCTGGAATATCGTATACATGATACAGCTGAGTGAGCGGCCCTCCAGCGTACTCCG
ACACACAAAAGATGTTTCAGGCCCCATTTGGCTCAAGTCTACTCCAGTTCCTGTGCTAAGAGG
TCCTCTTGATCTGTCTTGACTCCAAACCGTGGGACGTTGGTGTTCAGCTTCAGTGTGTAC
CAGCTTTTTTCAGCCCTGTGATCTGAGACATGGGCTGGGACTGTCNCACCACTGACGGCGGGG
GCTGCTGGAAGGCTCTCTGCCAAGGTGTGGGTGAGGGGATCCCCCACTTCATTTCTGCTTGT
TTTGATATTAAAGGTCTTCCCTGCCGGACNGCAAATGCCTGGTCTTCTTCCTCTAAAGGGTT
TGAAACCTGGGGACCCCTTNTAAAAAACCTTTTCCTGGGGAAGGTGGGTGGGCCCCCCTTGG
GGGTCTTAAGGGATGGTTATGGGGA
```

Table 4.14: Sequencing Results (Primer 15-1 in PDE4B)

[illegible]

Table 4.15: Sequencing Results (Primer 23-1 in PDE4B)

```

>23-1_PDE4B_BrainForwardPrimer
TATCNTAGCAATGAATAGAGTATGAACGGAGATGGGCTTGCTGTGGATTAGCAAATACCGCCC
ATTTGGAAGGCTTTGTCTTTTCTAAGTTTTGGTTCTTTAACCACCGGAGCTGAGAGCTGCGGA
GGTTTCCTGATCCTCTGGAGACCGCTCTTACAGAACTGAATTCTTTCAAAGGGATTTGTGGAT
TGTGGCAAGAAGAGCTTCCAAAATGCCTGAGGCAAACCTATTTGTTATCTCTATCTTGGGGTTA
TATCAAGTTCAAAAGGATGCTAGAACCGGGAGCTGACACACCTCTCAGAGATGAGCAGATCAG
GGAACCAAGTGTCTGAATACATTTCTGAACACGTTCTTAGACAAGCAGAACGATGTGGAAATCC
CATCTCCCACCCAGAAGGACAGGGAGAAGAAGAAGCAGCAGCTCATGACCCAGATAAGTG
GAGTGAANAACTGATGCACAGCTCAAGCCTGAACAACCACAAGCATCTCACGCTTA

>23-1_PDE4B_BrainReversePrimer
TNGNACTGATGTAATTATACGGCTTGAGCTGTAGCATCAGTTTCTTCACTCCACTTATTGCTA
GGGTCATGAGCTGCTGCTTCTTCTTCTTCTCCCTGTCCTTCTGGGTGGGAGATGGGATTTCCA
CATCGTTCTGCTTGTCTAAGAACGTGTTTCAAATGTATTACAGACACTTGGTTCCCTGATCTGC
TCATCTCTGAAGAGGTGTGTCAGCTCCCGGTTTCCAGCATCCTTTTGAAGTTGATATAACCCCAA
GATAGAGATAACAAATAGTTTGCCTCAGGCATTTTGGAAAGCTCTTCTTGCCACAATCCACAAA
TCCCTTTGAAAGAATTTCAGTTCTGTAAGAGCGGTCTCCAGAGGATCAGGAAACCTCCGCAGCT
CTCAGCTCCGGTGGTTAAAGAACCAAAACCTTAGAAAAGACAAAGCCTTCCAAATGGGCGGTAT
TTGCTAATCCACAGCAAGCCCATCTCCGTTTACTACTCTATTCTTGCAAGAAGCCTATGGGCTC
CC

>23-1_PDE4B_CerebellumForwardPrimer
CNGCTATGCTAGGCTTTGATCAGAGTATGAACGGAGATGGGCTTGCTGTGGATTAGCAAATAC
CGCCCATTTGGAAGGCTTTGTCTTTTCTAAGTTTTGGTTCTTTAACCACCGGAGCTGAGAGCT
GCGGAGGTTTCTCTGATCCTCTGGAGACCGCTCTTACAGAACTGAATTCTTTCAAAGGGATTTG
TGGATTGTGGCAAGAAGAGCTTCCAAAATGCCTGAGGCAAACCTATTTGTTATCTCTATCTTGG
GGTTATATCAAGTTCAAAAGGATGCTGAACCGGGAGCTGACACACCTCTCAGAGATGAGCAGA
TCAGGGAACCAAGTGTCTGAATACATTTCTGAACACGTTCTTAGACAAGCAGAACGATGTGGAA
ATCCCATCTCCCACCCAGAAGGACAGGGAGAAGAAGATAAAGCAGCAGCTCATGACCCAGATA
AGTGGAGTGAANAACCTGATGCACAGCTTCATGCCTGAACAACCACAAGCTTCTCCCGCNAAA
GAATAA

>23-1_PDE4B_CerebellumReversePrimer
TACTGTAGGGTANTTATAGCGCTTGAGCTGTAGCTCAGGTTTCTTCACTCCACTTATCTAGGG
TCATGAGCTGCTGCTTCTTCTTCTTCTCCCTGTCCTTCTGGGTGGGAGATGGGATTTCCACAT
CGTTCTGCTTGTCTAAGAACGTGTTCAAATGTATTACAGACACTTGGTTCCCTGAATCTGCTC
ATCTCTGAAGAGGTGTGTCAGCTCCCGGTTTCCAGCATCCTTTTAGAAGTTGAATATAACCCCAA
GATAGAGATAACAAATAGTTTGCCTCAGGCATTTTGGAAAGCTCTTCTTGCCACAATCCACAAA
TCCCTTTGAAAAGAATTTCAGTTCTGTAAGAGCGGTCTCCAGAGGATCAGGAAACCTCCGCAGC
TCTCAGCTCCGGTGGTTAAAGAACCAAAACCTTAGAAAAGACAAAGCCTTCCAAATGGGCGGTA
TTTGCTAATCCACAGCAAGCCCATCTCCGTTTACTACTCTATTCTTGCAAGAAGCCTATGGGCT
CCC

>23-1_PDE4B_RetinaForwardPrimer
AATCTAAGANCTTTAGACATATAGGAACGGAGTAGGGCTTGCTGTGGATTAGCAAATACCGCC
CATTTGGAAGGCTTTGTCTTTTCTAAGTTTTGGTTCTTTAACCACCGGAGCTGAGAGCTGCGG
AGGTTTCCTGATCCTCTGGAGACCGCTCTTACAGAACTGAATTCTTTCAAAGGGATTTGTGGA
TTGTGGCAAGAAGAGCTTCCAAAATGCCTGAGGCAAACCTATTTGTTATCTCTATCTTGGGGTT
ATATCAAGTTCAAAAGGATGCTAAACCGGGAGCTGACACACCTCTCAGAGATGAGCAGATCAG

```



```
GGAACCAAGTGTCTGAATACATTTCTGAACACGTTCTTAGACAAGCAGAACGATGTGGAAATCC  
CATCTCCCACCCAGAAGGACAGGGAGAAGAAGAAGCAGCAGCTCATGACCCAGATAAGTG  
GAGTGAAGAACTGATGCACAGCTCAAGCCTGAACAACACAAGCATCTCACGCTTA
```

```
>23-1_PDE4B_RetinaReversePrimer
```

```
TTTTCGGCTTGAGCTGTAGCATCAGTTTCTTCACTCCACTTATCTAGGGTCATGAGCTGCTGC  
TTCTTCTTCTTCTCCCTGTCCTTCTGGGTGGGAGATGGGATTTCACATCGTTCTGCTTGTCT  
AAGAACGTGTTTCGAAATGTATTTCAGACACTTGGTTCCTTGATCTGCTCATCTCTGAAGAGGTG  
TGTCAGCTCCCGGTTTCAGCATCCTTTTGAACCTTGATATAACCCCAAGATAGAGATAACAAATA  
GTTTGCCTCAGGCATTTTGAAGCTCTTCTTGCCACAATCCACAAATCCCTTTGAAAAGAATT  
CAGTTCTGTAAGAGCGGTCTCCAGAGGATCAGGAAACCTCCGCAGCTCTCAGCTCCGGTGGTT  
AAAGAACCAAACTTAGAAAAGACAAAGCCTTCCAAATGGGCGGTATTTGCTAATCCACAGCA  
AGCCCATCTCCGTTCTACTCTATTCTTGCAAGAAGCCTATGGGCTCCCTGCC
```

4.9 Discussion

Computational mapping of publicly available rat ESTs to genomic regions for the PDE4A, PDE4B, PDE4C and PDE4D genes yields more than 25 previously unreported exonic regions. In actuality, the number may be smaller since a number of these regions could be non-overlapping ESTs that would be merged into a single exon if the complete sequence was known. The majority of the novel found patterns are located in the N-terminal region (32 patterns) with additional seven occurring in the UCR1-UCR2 linker region, four mapping to the UCR1 region, six occurring in the UCR2 region, two occurring in the linker region between UCR2 and the catalytic core, and four occurring in the catalytic core itself. The locations of these previously undetected patterns is in line with previous research showing that the aminyl end of the protein is more susceptible to variability, producing long and short isoforms of PDE4 proteins (157;163). However, this study indicates additional exons in the carboxyl end, which was typically thought to be highly conserved due to the presence of a catalytic region. Molecular techniques verify that three of these exons are present with a degree of tissue specificity.

A potential shortcoming of looking at ESTs for alternative splicing is that the 5' ends of the mRNAs are underrepresented, due to the fact that the majority of EST libraries are constructed using poly-T primers that anneal to the 3' end of the mRNAs. As a result, a number of exons in the variable region of the PDE4 family of genes are potentially missed.

CHAPTER V

COMPUTATIONAL METHOD FOR PSEUDOGENE DETECTION AND CHARACTERIZATION

5.1 Pseudogenes

Genes are the basic unit to transfer hereditary information. They provide structure, function and regulation to a biological system. The genetic blueprint of an organism can be understood by studying the architecture of these genes. Pseudogenes, on the other hand, are regions sharing a level of sequence homology to functional genes that have somehow lost their ability to operate as genes. Pseudogenes may contain stop codons²⁷, have frame shifts²⁸ and a lack of transcription. However, they might retain gene-like features, such as promoters, CpG islands²⁹ and splice sites, and are therefore of interest to biologists who wish to study the effects of evolutionary processes.

As discussed in Section 1.6, a gene must go through several steps from a genetic DNA sequence to a fully-functional protein: transcription, pre-mRNA processing, translation and protein folding (Figure 5.1). If any of these steps fails, the sequence may be considered nonfunctional. The most commonly identified disablements are stop codons and frame shifts, which almost universally stop the translation of a functional protein product.

²⁷ Stop codons are TAA, TAG and TGA.

²⁸ A frame shifting is a genetic mutation that inserts or deletes a number of nucleotides that is not evenly divisible by three from a DNA sequence.

²⁹ CpG islands are short genomic regions that contain a high frequency of CG dinucleotides than in other regions. 'p' indicates that 'C' and 'G' are connected by a phosphodiester bond.

Figure 5.1: Central Dogma.

Figure 5.2: Pseudogene formation.

5.1.1 Processed Pseudogenes

Processed pseudogenes are generated by retrotransposition, which involves reverse transcription of an mRNA transcript and subsequent re-integration into genomic DNA. For human chromosome 22, 19% of the coding sequence corresponds to pseudogenes, 82% of which are processed pseudogenes (176;177). Processed pseudogenes represent the vast majority of inactivated gene sequences found in the human genome.

There are three basic features of a processed pseudogene. They are

1. A lack of non-coding intervening sequences (introns),
2. The presence of a poly-A tail at the 3' end, and

3. Homological extension (flanking direct repeats, which are associated with movable genetic elements) (176;178).

In the human genome, there are a number of different estimations for the total number of processed pseudogenes. Zhang et al. predicts ~7,800 processed pseudogenes (179) while Torrents *et al.* estimates there are ~13,800 (180). Ohshima *et al.* report 3,664 processed pseudogenes in the human genome and estimate a total number ~7,000 processed pseudogenes, based on an estimation of ~35,000 human genes (181-183).

5.1.2 Unprocessed Pseudogenes

In the process of duplication, genetic sequences can go through various modifications, including mutation³⁰, frame shifting, insertions and deletions. Any significant modification during the translational or transcriptional stage could result in a loss of genetic function. Hence, if the duplication of a gene is incomplete, the new sequence could be a pseudogene. Those pseudogenes derived from duplication are called unprocessed pseudogenes (65;176;184). It arises when a cell is replicating its own DNA and inserts an extra copy of a gene into a genome in a new location (185).

Unprocessed pseudogenes may have introns retained (as shown in Figure 5.2). They can be a complete or partial copy from the parent gene. Sometimes, the unprocessed pseudogenes contain an extra copy of the gene. The unnecessary extra copy “could accumulate mutations without harming the organism” (178).

³⁰ A mutation is a change of the DNA sequence within a gene or chromosome of an organism resulting in the creation of a new character or trait not found in the parental type. Mutation can also mean the process by which such a change occurs in a chromosome, either through an alternation in the nucleotide sequence of the DNA coding for a gene or through a change in the physical arrangement of a chromosome.

In addition, the unprocessed pseudogene can be created secondarily from pre-existing processed pseudogenes. In other words, the parental source gene may be intronless. In this case, the duplication occurred after transcription (176).

5.1.3 Disabled Pseudogenes

A third type of pseudogenes, known as disabled or unitary pseudogenes, are becoming more widely studied (186;187). When various mutations occur, they can disrupt transcription or translation of a gene. Moreover, if these mutations become fixed in a population, it is possible the gene may become nonfunctional or deactivated in a mechanism similar to unprocessed pseudogenes. The difference between the two is that disabled pseudogenes are not duplicated before becoming disabled (188).

5.1.4 Functional Pseudogenes

Up to this point, pseudogenes have been defined as having no function in Sections 1.8.3 and 5.1.1-5.1.3. However, in 2003, Hirotune *et al.* in Japan found that a retrotransposed pseudogene whose transcript plays a *trans*-regulatory role in the expression of its homologous gene, *Makorin1*. They suggested a general model that pseudogenes may play an important biological role (189;190). In 2004, Hirotune and his team reported that about 2-3% of human processed pseudogenes are expressed using the strict criteria based on their algorithm and wet laboratory experimentation (191). However, their result has been strongly contested by Gray's team due to the inability to reproduce Hirotune's experiment (192). Therefore, the functional role of pseudogenes is of current interest.

5.1.5 Motivations for Pseudogene Detection and Characterization

Computational sequence analysis can be complicated by the presence of pseudogenes. For instance, it is possible that a researcher wants to amplify a certain gene by using polymerase chain reaction (PCR) but accidentally and simultaneously amplifies a pseudogene due to their share similar sequences. Another example is in *ab initio*³¹ gene prediction where processed pseudogenes are often misidentified as real genes or exons. Identification of pseudogenes will help to improve the accuracy of gene prediction (183). In addition, identifying pseudogenes may lead to a better understanding of gene evolution.

5.2 Current Automatic Pseudogene Detecting Methods

5.2.1 Harrison's Approach

Harrison *et al.* from Yale University developed a pseudogene annotation pipeline in 2001 (193). They used *Caenorhabditis elegans* genome to test their seven-step process. First, they downloaded annotated pseudogene sequences from Sanger Centre and analyzed those sequences. They found about 6% of the annotated pseudogenes are not detectable when considering simple disablement, such as a frameshift or premature stop codon. Second, they aligned protein sequences (Wormpep18, SCOP and PROTOMAP) to the genome sequences using FASTA in order to find potential pseudogenes. All six-frame sequences (three forward and three backward strands) were analyzed. Third, alignment results were filtered for overlapping matches in genomic sequences, if the size

³¹ In computation, *ab initio* means an extra, transform, load tool used to manipulate data. In Bioinformatics, *ab initio* is a term used to define methods for making predictions about biological features using only a computational model without extrinsic comparison to existing data. In this context, it may be sometimes interchangeable with the Latin term *de novo*.

of the overlap is over 30 nucleotides. Fourth, to avoid over-counting for worm protein hits, the initial matches were further filtered for adjacent matches. Fifth, the potential pseudogenes were filtered for overlap with any other annotations in the Sanger Centre files such as exons of genes, tandem or inverted repetitive and transposable elements. Sixth, they refined the data for additional repeat elements and any match to proteins. Seventh, they increased the threshold e-value from 0.01 to 0.001 (193).

In 2002, using the same methodology, Harrison *et al.* released their results for detecting pseudogenes in human chromosome 21 and 22. They found 77 processed pseudogenes on chromosome 21 and 112 on chromosome 22 (194). For unprocessed pseudogenes, they found 72 on chromosome 21 and 123 on chromosome 22 (194).

5.2.2 Sakai's Approach

In 2003, Sakai *et al.* from Japan introduced a method to detect processed pseudogenes based on cDNA mapping to the human genome (195). Their methods were divided into two sections. The first section is the cDNA mapping. They aligned all cDNA sequences against human genome using `blastn`. Then they extracted corresponding genomic regions for each query sequence. Next, they set up cut values of $\geq 95\%$ identity and $\geq 80\%$ coverage to filter those sequence hits based on the `est2genome`.

The second stage of the experiment is pseudogene detection. All the candidate sequences collected from the first stage were classified into six classes according to the way in which the introns are processed (Figure 5.3) before checking the 3' terminator.

Figure 5.3: Flow chart of Sakai *et al.* method for processed pseudogene detection.

Sakai and his team found 6,549 possible processed pseudogenes using 113,196 human cDNA with their automated pipeline including novel intron-containing pseudogenes. They believed their approach will help aid the accuracy of gene prediction (195).

5.2.3 PPFINDER

PPFINDER was released in 2006 to find processed pseudogenes (183). The purpose for the program is to improve the accuracy of gene prediction methods, if processed pseudogenes can be identified. A gene model was first established in order to collect the gene information. Two methods were used in order to find pseudogenes: intron location and conserved synteny. In the intron location method, it started to align pre-established gene model to cDNA database using `blastn`. The best scoring hits were

kept. Then, these transcripts were further aligned to genome locus. If the transcript hits more than one location in the genome, the transcript will be marked as pseudogene-derived for further testing. The segments of predicted gene will only hit themselves in the genome, if they are not pseudogene-derived. But if they are pseudogene-derived, the best hit score will be with the parental gene location. The other hit locations in the genome can be potential pseudogenes.

In the synteny method, the candidate pseudogene-derived sequences were split into exons and translated into amino acids. Then these translated sequences were aligned to the protein database using `blastp`. If the sequence identity of the hit is $\geq 65\%$ and the length of the hit is at least 10 amino acids, these sequences were kept. Then the sequences were examined if their hit location is the same as the gene model. If the answer is no, the information of conserved syntenic region will be recorded. Once again, they aligned the original created gene model to the recorded information to find orthologous region. If the orthologous regions were found, the gene model is said to contain a pseudogene.

However, if it is a single-exon gene, without locating introns, the sequence might be counted as a repeat and would be failed to identify as pseudogene-derived sequence (183).

5.2.4 PseudoPipe

Zhang *et al.* from Yale University proposed an automated pseudogene identification pipeline in 2006 (196). Their method was based on Harrison's approach (both Harrison and Zhang were post-docs training in Gerstein's laboratory at Yale University). This pipeline began by aligning protein sequences to genomic sequences

using `tblastn`. Then the sequences overlapping with the gene sequences in ENSEMBL database were removed. Smith-Waterman was used to optimize the alignment to merge and extend candidate sequences. Fourth, false positive, repeats, low complexity sequences and potential functional gene candidates were removed. The pseudogene candidate is considered a false positive if its BLAST e-value is $\leq 10^{-10}$ or an amino acid sequences identity is $\leq 40\%$. Finally, sequences are separated into different classes – retrotransposed pseudogenes, duplicated pseudogenes (or unprocessed pseudogenes in our definition), or pseudogeneic fragments based upon exon structure (179;182;196;197).

5.2.5 Pseudogenes Analysis in ENCODE Project

In Section 1.7.4, the ENCODE project was discussed. It brought dozen of laboratories together to participate to analyze and identify the functional elements in the human genome. Studies were focused on transcripts and gene models, protein-binding sites, epigenetic markers, and promoters and enhancers, evaluating the existing methods and creating new methods if necessary. One group focused on methods for detecting pseudogenes.

As a subgroup of ENCODE, the pseudogene research team focused on establishing methods to decide consensus annotation, analyze transcription and study evolution. The first goal was to “obtain an accurate list of pseudogenes in order to facilitate the creation of a comprehensive catalog of structural and functional elements in the ENCODE regions” (47;56). They evaluated five developed methods: GIS-PET (198), HAVANA (199), PseudoPipe (55;196), pseudoFinder (unpublished), and retroFinder (200;201).

The pseudogene research team found that all five methods detect pseudogenes based on sequence similarity to “at least one entry in a collection of query sequences representing known human genes (referred to as the parent genes).” The main two differences among these five methods are in (1) type of queries, either nucleotides or proteins, used to search for pseudogenes and (2) strategies used to assess a sequence’s coding potential and methods for distinguishing processed from unprocessed pseudogenes (56).

The second task was to establish a consensus procedure including manual curation in order to get the correct and reliable list of pseudogenes. The research team only considered those pseudogenes that match known proteins from the UniProt database (202).

After evaluating the five computational methods, they characterized the sequences they found and sequenced these pseudogene transcripts. They realized that in the ENCODE gene-rich regions, more pseudogenes were found. In addition, most sequences are decayed gene copies that contain frame shift mutations (56).

5.3 Characteristics for Pseudogene Detection

In the previous sections, different types of pseudogenes and the current computational methods have been discussed. Based on the biological factors and other’s work, there are some features that need to be investigated when evaluating pseudogene candidate sequences. They are start and stop codons, poly-A signals and poly-A tails, G+C composition, and mutational events. Each of these features is considered in a newly developed method to score sequences for their likelihood of being a pseudogene. The rest

of Section 5.3 covers in detail the features considered for the method constructed in Section 5.5.

5.3.1 Start Codon

When a fully-functional protein is translated from a genetic sequence, it goes through transcription, pre-mRNA processing, translation and protein folding (Figure 5.1). By default, ATG, or AUG in RNA sequences, serves as a start codon or initiator to translate genes to proteins (203). There are rare cases in eukaryotes initiated by codons other than ATG/AUG (204-209), such as GTG/GUG. The standard code currently allows initiation from TTG/UUG and CTG/CUG in addition to ATG/AUG (203).

When detecting pseudogenes, locating a conserved or mutated ATG in an open reading frame is a good starting point. It is possible that ATG will be located in a different or a later place other than the first position in a sequence. The alternative patterns of ATG from Table 5.1 are examined.

Table 5.1: Start Codon Alternative Patterns and Their Scores

Main pattern		Alternative patterns				
ATG	1.00	ATN	ATA	ATC	ATT	0.25
		ANG	AAG	ACG	AGG	0.25
		ANN	AAN	ACN	AGN	0.0625
			AAA	ACA	AGA	
			AAC	ACC	AGC	
			AAT	ACT	AGT	
		NTG	CTG	GTG	TTG	0.25
		NNG	CNG	GNG	TNG	0.0625
			CAG	GAG		
			CCG	GCG	TCG	
CGG	GGG		TGG			
NTN	CTN	GTN	TTN	0.0625		
	CTA	GTA	TTA			
	CTC	GTC	TTC			
	CCT	GGT	TTT			
NNN					0.015625	

5.3.2 Stop Codons

A terminator is present to tell the cell to end the translation process and release the amino acid chain. TGA, TAG and TAA (or UGA, UAG, and UAA in RNAs) are three well-known stop codons (210-212). Stop codon insertion and mutation is one of the common features differentiating pseudogenes from regular genes. Pseudogenes are caused by either retrotranscription or duplication. Frame shift, mutation, insertion or deletions may cause the terminating signal relocate earlier in the sequences. In such a case, in the open reading frame, the premature stop codon causes translation to terminate. Finding premature stop codons is the most common feature to consider a sequence being a pseudogene sequence. Two conditions are considered:

- Presence of a start codon near the beginning of a sequence

- Presence of a stop codon located downstream the start codon

Besides the most widely found stop codons, TGA/UGA, TAG/UAG, and TAA/UAA, an additional thirteen mutational patterns were inspected as well (Table 5.2).

Table 5.2: Stop Codon Alternative Patterns and Their Scores

Main pattern		Alternative patterns				
TGA	1.00	TGN	TGC	TGG	TGT	0.25
TAG	1.00	TNG	TCG		TTG	0.25
TAA	1.00	TAN	TAC		TAT	0.50
		TNA	TCA		TTA	0.50
		TNN	TCN		TTN	0.25
			TCC		TTC	
			TCT		TTT	
		NAA	AAA	CAA	GAA	0.25
		NAG	AAG	CAG	GAG	0.25
		NGA	AGA	CGA	GGA	0.25
		NNA	ANA	CNA	GNA	0.0625
			ACA	CCA	GCA	
			ATA	CTA	GTA	
		NNG	ANG	CNG	GNG	0.0625
			ACG	CCG	GCG	
			AGG	CGG	GGG	
				CTG	GTG	
		NAN	AAN	CAN	GAN	0.0625
			AAC	CAC	GAC	
			AAT	CAT	GAT	
		NGN	AGN	CGN	GGN	0.0625
			AGC	CGC	GGC	
			AGT	CGT	GGT	
		NNN				0.015625

5.3.3 Poly-A Signals and Poly-A Tails

Polyadenylation is the covalent linkage of a poly-A tail to an mRNA molecule in order to stabilize the molecule so a mature mRNA will be ready for translation and produce an amino acid. The process starts by finding a cleavage site which is

characterized by the presence of a poly-A signal sequence near the cleavage site, mostly commonly found to be AATAAA (or AAUAAA in RNA). When the mRNA has been cleaved, a number of adenine (A) residues (50-250) will be attached in the 3' end at the cleavage site. The reaction is catalyzed by polyadenylate polymerase (213).

When pseudogenes are formed through retrotranscription or duplication, the incompletely translated sequences may be re-inserted into the genomic sequences. Those sequences may contain a poly-A site, just like processed mRNAs. It is also possible they have a number of adenines (A) in the 3' end of the sequence. In fact, most processed pseudogenes have shown the presence of a poly-A tail in the 3' end.

Table 5.3: Poly-A Signal Patterns

polyA site	% sites	polyA site	% sites
AATAAA	58.2	AATAGA	0.7
ATTAAA	14.9	AAAAAG	0.8
AGTAAA	2.7	ACTAAA	0.6
TATAAA	3.2	AAGAAA	1.1
CATAAA	1.3	AATGAA	0.8
GATAAA	1.3	TTTAAA	1.2
AATATA	1.7	AAAACA	0.5
AATACA	1.2	GGGGCT	0.3

Abbreviations: poly-A, polyadenine;
%, percentage. These 16 poly-A sites are collected from Table 2 in (214).

AATAAA is the most common poly-A signal in human genome, found in close to 60% of gene sequences. ATTAAA is also relatively common, occurring 14.9% of the time. There are fourteen other patterns been identified as poly-A signals (Table 5.3). Based on their occurrence rate, different scores were assigned for each poly-A signal patterns.

For poly-A tails, finding a large number of adenine (A) residues in a sequence is too difficult since these regions can also be counted as low complexity. Instead, we detect regions with a run of at least ten adenines, increasing the score according to the total number of A's found. If a candidate sequence owns a poly-A signal and a poly-A tail of length ten or more, the possibility being a pseudogene is greatly increased.

5.3.4 G+C Composition

In Harrison *et al.*'s research, they found the pseudogenes have higher G+C composition than the background genomic DNA in general (215;216). The G+C composition ranges from 36 to 54 percent in the sequences (217). Therefore, the candidate sequences will be reviewed to see how many G's and C's are involved in the sequences.

5.3.5 Similar, Synonymous vs. Nonsynonymous Mutations

When two sequences are counted as homologous, they share a certain percentage of similarity. Both of them have originated from a common ancestral sequence but accumulated various mutations. Figure 5.4 shows the types of mutations occurring an example pair of sequences. Although there are three differences between these two sequences, a single substitution, sequential substitution, coincidental substitution, parallel substitution, convergent substitution and back substitution have also occurred.

In Section 1.4, the genetic code was discussed. The $4 \times 4 \times 4$ or 64 different combinations of codons from A, C, G and T represent the twenty common amino acids. Based on Table 5.4, we can translate these two descendant sequences into amino acids.

Although there are only three nucleotides changed in each descendant sequence, some of the amino acids have been changed.

Figure 5.4: Mutations occurring in two homologous sequences.
Image adopted and modified from Figure 3.6 in (218).

Table 5.4: The Genetic Code (RNA to Amino Acids)

		Second letter				
		U	C	A	G	
First letter	U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	Third letter
		UUC	UCC	UAC	UGC	
		UUA Leu	UCA	UAA Stop	UGA Stop	
		UUG	UCG	UAG Stop	UGG Trp	
	C	CUU Leu	CCU Pro	CAU His	CGU Arg	
		CUC	CCC	CAC	CGC	
		CUA	CCA	CAA Gln	CGA	
		CUG	CCG	CAG	CGG	
	A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	
		AUC	ACC	AAC	AGC	
		AUA	ACA	AAA Lys	AGA Arg	
		AUG Met	ACG	AAG	AGG	
	G	GUU Val	GCU Ala	GAU Asp	GGU Gly	
		GUC	GCC	GAC	GGC	
		GUA	GCA	GAA Glu	GGA	
		GUG	GCG	GAG	GGG	

In descendant sequence (1), the 5th, 7th, 9th and 11th bases have been changed after mutations. Although it does not look like a lot of changes in the nucleotide acid sequence, the amino acids which contain these four mutational nucleotides respectively have been changed (Figure 5.5).

	Thr				Glu				Arg				Asn				Ala		
	A	C	T		G	A	A		C	G	T		A	A	C		G	C	T
					↓				↓		↓		↓						
	A	C	T		G	G	A		G	G	A		A	T	C		G	C	T
base	1	2	3		4	5	6		7	8	9		10	11	12		13	14	15

Figure 5.5: Comparison between ancestral sequence and descendant sequence (1).

In descendant sequence (2), there are also three mutations locating in the 2nd, 7th, 9th and 11th bases. Two of the three amino acids which contain the three mutational nucleotides respectively have been changed (Figure 5.6).

	Thr				Glu				Arg				Asn				Ala		
	A	C	T		G	A	A		C	G	T		A	A	C		G	C	T
	↓								↓				↓						
	A	A	T		G	A	A		A	G	A		A	T	C		G	C	T
	Asn				Glu				Arg				Ile				Ala		
base	1	2	3		4	5	6		7	8	9		10	11	12		13	14	15

Figure 5.6: Comparison between ancestral sequence and descendant sequence (2).

The 7th and 9th nucleotides changed in descendant sequence (2) can be counted as synonymous mutations because the amino acid does not change. However, the other mutational nucleotides shown in Figures 5.5 and 5.6 that change the amino acids have to be studied further in order to distinguish if they produce nonsynonymous or similar synonymous mutation.

Those mutations at the nucleic level that result in a similar but not identical amino acid are called similar synonymous mutations. Although the mutational nucleotide does change the amino acid, the functionality of the amino acid is close to the original one. But if mutations at the nucleic level change the amino acid to something unrelated, it is called a nonsynonymous mutation. To find out how similar or how close the amino acids are between each other, the PAM250 matrix generated by Dayhoff (97) and the BLOSUM62 matrix generated by Henikoff (219;220) are the two matrices widely used. In WU BLAST, BLOSUM62 matrix is chosen by default when comparing protein sequences. If

the comparison results a same amino acid, it will display the amino acid. But if the comparison returns a close amino acid, it will give a plus '+' sign.

Subject	G A G E	A A T N	C C C P	A A G K	A A G K	T A C Y	A T C I
blastx	E		P	K	K	+	I
Query	G A G E	A C T T	C C C P	A A G K	A A G K	C A C H	A T C I
base	1 2 3	4 5 6	7 8 9	10 11 12	13 14 15	16 17 18	19 20 21

Figure 5.7: Similar synonymous mutation vs. nonsynonymous mutation.

Subject, subject sequences (nucleotide and amino acid); blastx, results returned by blastx; Query, query sequences (nucleotide and amino acid).

In Figure 5.7, the nucleotides located at the 5th and 16th bases have mutated. Both amino acids which contain these two mutational nucleotides are changed as well. According to the blastx result, the amino acid changed from N (Asn) to T (Thr) is not related (blank in the blastx result row). The nucleotide substitution is a nonsynonymous mutation. But the amino acid changed from Y (Tyr) to H (His) is related (plus '+' sign in the blastx result row). Thus, the nucleotide substitution is called a similar synonymous mutation.

To evaluate the pseudogene candidates, determining the number of similar synonymous mutations (the amino acid is changed to a close amino acid), synonymous mutations (the amino acid is not changed) and nonsynonymous mutations (the amino acid is changed to a unrelated amino acid) will be calculated.

5.4 Sequence Data

All the approaches discussed in Section 5.2 begin pseudogene analysis by aligning sequences to genomic regions. Besides Sakai *et al.* who use cDNA sequences, all other approaches use protein sequences or RefSeq sequences for alignments.

Similar to the methods that have been discussed in Section 5.2, the pseudogene detection and characterization in this project begins with sequence alignment. Rather than using cDNA, RefSeq or protein sequences in as other approaches, EST sequences were chosen in this project. Compared to cDNA, RefSeq or protein sequences, ESTs are relatively short, ranging from 300 to 600 bp in length. The size of cDNA, RefSeq or protein sequences could range from 1000 to 4000 bp. Aligning ESTs to genomic sequences will return more hits from different locations of genomic regions due to their smaller size. It is possible to return the high scoring pairs (HSP) from the genomic locations that are never reported before from the previous methods due to longer query sequences.

Although it will increase the amount of data for further analysis, it is possible to provide more candidate locations in genomic regions as pseudogenes. Moreover, with the choices of alternative splicing (which has been studied in Chapter 4), it will bring an additional possibility to locate genomic hits other than the parental hit itself.

In the current approaches, EST sequences have not been used for identifying pseudogenes. Hence, using ESTs for initial alignment for searching and determining candidate pseudogenes provides a novel source of data.

Figure 5.8: Process for pseudogene detection.

Pseudogenes can be detected in the steps out-lined in Figure 5.8. After downloading the necessary sequence files, each sequence will go through data preprocessing, sequences alignment, clustering and pseudogene detection. The detailed information for the methodology will be discussed in Section 5.6.

Expressed sequence tags (ESTs) were downloaded from GenBank/dbEST release 158.0, dated 4-18-2007 (<http://www.ncbi.nlm.nih.gov/dbEST/>) (72) for the purpose of detecting and characterizing pseudogenes. RefSeq sequences were downloaded from NCBI/RefSeq release 22, dated 3-13-2007 (<http://www.ncbi.nlm.nih.gov/RefSeq/>) (73-76). Genomic regions were downloaded from UCSC Genome Browser (<http://genome.ucsc.edu>) (77-81).

5.5 Methodology

5.5.1 Data Preprocessing

EST sequences downloaded from GenBank/dbEST are parsed to include only those from the species of interest. Raw EST sequences are masked for repetitive elements using RepeatMasker (87;88). Low complexity regions for ESTs are then masked using the DUST (70) filter available from the wuBLAST software (70;167).

5.5.2 ESTs to Genome Alignment

After organism sequences are filtered, ESTs are aligned to genomic regions using `wublastn` with parameters `W=15 topComboN=5 -nogaps -gspmax=100 -hspmax=500 filter=dust -warning -notes -error -links`. The results run by `blastn` are then filtered by a Perl script to parse out the necessary information (Figure 5.9).


```
=gi|5555714|gb|AV175813.1|/gbest46.seq.gz AV175813 Yuji Kohara unpublished
cDNA: Strain N2 hermaphrodite embryo Caenorhabditis elegans cDNA clone yk487b3
3' (300 letters; record 79)
```

>chrIII	741	5971574	5971723	33	182	1	(1)-2	0.99	0.50	0.50
>chrIII	593	5972503	5972622	181	300	1	1-(2)	0.99	0.40	0.40
>chrIII	136	12875675	12875730	36	91	2	(3)-4	0.71	0.19	0.13
>chrIII	95	13163938	13163974	84	120	2	3-(4)	0.73	0.12	0.09
>chrIII	124	13114293	13114342	58	107	3	(5)	0.72	0.17	0.12
>chrII	123	12241150	12241215	46	111	1	(1)	0.65	0.22	0.14
>chrII	120	15033827	15033868	69	110	2	3-(2)	0.76	0.14	0.11
>chrII	97	1139631	1139653	44	66	2	(3)-2	0.91	0.08	0.07
>chrV	130	19475058	19475101	76	119	1	2-(1)-3	0.77	0.15	0.11
>chrV	103	8552104	8552129	39	64	1	(2)-1-3	0.88	0.09	0.08
>chrV	94	20038590	20038642	173	225	1	2-1-(3)	0.64	0.18	0.11
>chrIV	128	11169253	11169310	88	145	1	2-(1)-3	0.69	0.19	0.13
>chrIV	103	7401906	7401958	42	94	1	(2)-1-3	0.66	0.18	0.12
>chrIV	93	12955563	12955595	195	227	1	2-1-(3)	0.76	0.11	0.08
>chrIV	124	1160745	1160812	45	112	2	(4)-5	0.65	0.23	0.15
>chrIV	102	9483397	9483483	97	183	2	4-(5)	0.57	0.29	0.17
>chrIV	124	959701	959768	37	104	3	(6)	0.65	0.23	0.15

Figure 5.9: Sample blastn result for GI 5555714.

Column 1, chromosome; column 2, high scoring pair score; columns 3 and 4, begin and end locations in genomic regions; columns 5 and 6, begin and end locations in ESTs; column 7, group; column 8, links; columns 9, 10 and 11, percent identity, percent coverage and match value.

Each record starts with an equal sign '=' followed by a description of the EST. The following rows, starting a greater sign '>' represents a sequence hit, including the chromosome number, HSP score, begin and end locations of the hits in the genomic sequences, begin and end locations of the hit in EST, the group number, the links, the percent identity of the hit, the percent coverage of the hit and the match value of the hit.

Since pseudogenes have a high similarity to the genome sequence of origin and ESTs have a 2-5% sequence error rate (221), only sequences whose identified similarities are greater than or equal to 95% are reserved. Ideally, these sequences are closer to the target pseudogenes. However, the hit size has to be considered as well. If the HSP is greater or equal to 95% but hits fewer than 40 bases out of a 400 bp EST, the coverage is low.

The `blastn` computational results were filtered based on the following criteria: if it is a single splice hit, discard hits with length less than 50 bp. If it is multiple-splice hit, the sequence hit has exactly two splices, and the gap between two hits are over 1,000,000 bp, discard the record. Otherwise, measure the size of the hit with each splice and discard the record whose percent identity is less than 70%. Results are compared (single-splice hits and multiple-splice hits) and sorted by: accession number, chromosome number and genomic begin location. A flowchart of this process is provided in Figure 5.10.

As discussed in Sections 1.8 and 4.1.3 to 4.1.5, a gene sequence is derived from the combination of exons after the introns are removed. Those highly similar sequences which have been filtered might be the splices of genes themselves. Therefore, additional sequence comparison is needed. The set of spliced nucleotide sequences will be aligned to an unspliced genomic DNA sequence. Through this process, beginning and ending locations of exons and introns will be discovered.

In addition, GT/AG and CT/AG are commonly found at exon/intron boundaries known as donor and acceptor sites. Once the beginning and ending locations of exons and introns are known, the splice patterns of the gene can be detected. Most introns begin with GU (or GT in DNA) and end with AG patterns. With this information, the exact boundary between any two exons can be found by mapping ESTs back to the genome. However, the frequency of GU-AG combinations of bases is fairly common, occurring on an average about 1/16 of the time. A number of programs help distinguish these combinations (222).

Figure 5.10: Flow chart for filtering blastn results.

In Section 3.3.5, EXALIN (118) was discussed as a powerful method for determining sequence similarity and splice site. EXALIN can also align sequences blocked within a given range in the genome. After `blastn` results were filtered based on size and percent identity and sorted according to the genomic begin location, the hit is further refined to determine the leftmost and rightmost locations in genome in order to set up a searchable boundary in EXALIN. If the blocked range is over 1,000,000 bases, the search will be broken into pieces with a size of 500,000 bases. Otherwise, both boundaries will be extended to the closest thousand number. For instance, the hit after running `blastn` for GI 5555714 to *C. elegans* chromosome III were split into two pieces: 5971574-5971723 and 5972503-5972622. To use EXALIN for fine tuning, the search range will be 5971571-5972622. To extend both ends to the closest thousand number, it is actually comparing 5555714 against chromosome III between the genomic locations 5971000-5973000 when running EXALIN.

The comparison results calculated by EXALIN to find exon/intron junctions are then split into two groups: single-splice hit and multiple-splice hit. If it is a single-splice hit, sequences of length < 50 bp are discarded. If it is a multiple-splice hit, the number of segments, the left and right ends of each segment is recorded. All the single-splice and multiple-splice hits are merged and sorted in these order: accession number, chromosome number and genomic begin location.

Before processing fine tuning in sequence comparison, the searchable boundary for running EXALIN is based on the `blastn` result. Thus, if the two blocked boundaries in the same chromosome generated by `blastn` results are different, ideally EXALIN should return different computational outcomes. For example, `blastn` returns two

groups of HSP after comparing GI no. 18288550 against chromosome V for *C. elegans* (Figure 5.11). The boundaries found by blastn were 2230674-9303595 and 2230017-5705627, respectively, and the EXALIN blocked boundaries are 2230000-9304000 and 2230000-5706000 (Figure 5.12). Figures 5.12 and 5.13 show the results returned by EXALIN that is differing from what blastn has generated in Figure 5.11.

=gi 18288550 gb BJ128393.1 /gbest148.seq.gz BJ128393 unpublished oligo-capped cDNA library (722 letters)										
>chrV	212	2230674	2230764	31	121	10	(12)-15-16-13-14	0.70	0.13	0.09
>chrV	92	2713536	2713593	263	320	10	12-(15)-16-13-14	0.62	0.08	0.05
>chrV	86	8716204	8716231	331	358	10	12-15-(16)-13-14	0.79	0.04	0.03
>chrV	199	8971491	8971708	335	552	10	12-15-16-(13)-14	0.55	0.30	0.16
>chrV	97	9303573	9303595	562	584	10	12-15-16-13-(14)	0.91	0.03	0.03
>chrV	616	2230017	2230141	722	598	1	(18)-17-19	0.99	0.17	0.17
>chrV	2938	2230196	2230793	599	2	1	18-(17)-19	0.99	0.83	0.82
>chrV	99	5705583	5705627	78	34	1	18-17-(19)	0.69	0.06	0.04

Figure 5.11: Sample blastn result GI no. 18288550.
(partial result sorted by genomic begin location)

Column 1, chromosome number; column 2, high-scoring pair score; columns 3 and 4, begin and end locations in genomic regions; columns 5 and 6, begin and end locations in ESTs; column 7, group; column 8, links; columns 9, 10 and 11, percent identity, percent coverage and match value.

```

EXALIN [06-May-2005]
ARGS: -i 0 -s 3 -p /biodata/bin/exalin.linux24-i686/C.elegansSPM.par -A 2230000 -B 9304000
/biodata/elizabeth/elegansSeq/chrSeq/masked/ce2-Mar2004/chrV.fa
/biodata/elizabeth/testResults/elegansGenome/chr_vs_est/blast/4-18-07Re158extend/BJ128393.fa
T-SEQUENCE: gi|18288550|gb|BJ128393.1|/gbest148.seq.gz BJ128393 unpublished oligo-capped cDNA library
T-LENGTH: 722
G-SEQUENCE: chrV
G-LENGTH: 20922231
SCORE: 869.3 nats (1254.2 bits)
SPLICE-DIR: +
T-STRAND: -

EXON: 722 - 600 (2230017 - 2230139)    153.8   N/A   -3.71   100.00%
EXON: 599 - 2 (2230196 - 2230794)    740.8   -1.45   N/A    99.67%
SCORE: 884.2 nats (1275.6 bits)
SPLICE-DIR: -
T-STRAND: -

EXON: 722 - 600 (2230017 - 2230139)    153.8   N/A    4.47   100.00%
EXON: 599 - 2 (2230196 - 2230794)    740.8    5.22   N/A    99.67%

EXIT 0

```

Figure 5.12: Sample EXALIN result for GI 18288550.
(Blocked search range is 2230000-9304000.)

```

EXALIN [06-May-2005
ARGS: -i 0 -s 3 -p /biodata/bin/exalin.linux24-i686/C.elegansSPM.par -A 2230000 -B 5706000
/biodata/elizabeth/elegansSeq/chrSeq/masked/ce2-Mar2004/chrV.fa
/biodata/elizabeth/testResults/elegansGenome/chr_vs_est/blast/4-18-07Re158extend/BJ128393.fa
T-SEQUENCE: gi|18288550|gb|BJ128393.1|/gbest148.seq.gz BJ128393 unpublished oligo-capped cDNA library
T-LENGTH: 722
G-SEQUENCE: chrV
G-LENGTH: 20922231
SCORE: 869.3 nats (1254.2 bits)
SPLICE-DIR: +
T-STRAND: -

EXON: 722 - 600 (2230017 - 2230139)    153.8    N/A    -3.71    100.00%
EXON: 599 - 2 (2230196 - 2230794)    740.8    -1.45    N/A    99.67%
SCORE: 884.2 nats (1275.6 bits)
SPLICE-DIR: -
T-STRAND: -

EXON: 722 - 600 (2230017 - 2230139)    153.8    N/A    4.47    100.00%
EXON: 599 - 2 (2230196 - 2230794)    740.8    5.22    N/A    99.67%

EXIT 0

```

Figure 5.13: Sample EXALIN result for GI 18288550.
(Blocked search range is 2230000-5706000.)

After the fine tuning process using EXALIN, duplicate results are removed. Figure 5.14 gives a flow chart of the process to remove redundant records generated by EXALIN.

The records are further reviewed and split into two groups: records which hit single locations and records which hit multiple locations. If the same EST accession number hits multiple locations, either in the same or different chromosome, it will be assigned into the group whose records hit multiple locations. Otherwise, it is assigned into the group whose records hit single location. Because the sequence only hits the genome once, it is highly believed to be a gene itself or can be a disabled pseudogene. Disabled pseudogenes cannot be detected using this approach. The information will be saved for further usage. Records belonging to the group whose records hit multiple locations are potentially pseudogenes.

Figure 5.14: Flow chart for removing EXALIN redundant records.

5.5.3 Clustering

The purpose of clustering is to incorporate overlapping ESTs that tag the same transcript of the same gene in a single cluster. Thus, measuring the similarity between any two sequences is used to cluster sequences into suitable groups. It requires a simple Boolean measurement: accept (yes) or reject (no) two sequences in the same cluster.

Multiple-location records gathered from fine tuning are further sorted by chromosome number and genomic begin location. The sequence hits are then reviewed in a “DNA walking” method: the sequence hit is reviewed base to base. If the sequences overlap each other, they are grouped together and the leftmost and rightmost boundaries will be the left end of the first sequence and the right end of the second sequence (Figure 5.15). Both single-splice and multiple-splice hits are reviewed in the same format. The ESTs belong to the same cluster will be recorded in the table for information tracing. The only exception is when a distance between left and right boundaries of a record is over 500,000 bases will be assigned in an individual group. In such group, each segment of the hit will be examined by size of the segment, distance between segments, begin and end location of the hit in EST.

Figure 5.15: Clustering by DNA walking.

5.5.4 Pseudogene Detection and Characterization

After clustering EST hits into groups, each cluster will contain information pertaining to the left and right ends in genomic regions, length of the cluster and the number of ESTs in the cluster. Clusters containing gap(s) will be marked.

The left and right boundaries of each cluster are used to retrieve the corresponding genomic sequences. Each sequence is run against the RefSeq protein databases using `blastx` and processed to get scores for both forward and reverse directions. Each factor discussed in Section 5.3 will be examined:

1. Look for start codon (ATG),
2. Look for stop codon(s) (TGA, TAG and TAA) in open reading frames,
3. Search for poly-A signal,
4. Add up the number of adenine (A) in the poly-A tail if found,

5. Calculate G+C composition, and
6. Calculate values for similar synonymous mutations, synonymous mutations and nonsynonymous mutations.

5.6 Results

In order to demonstrate the effectiveness of this approach for pseudogene detection, a set of cytochrome³² gene and pseudogene sequences from the human genome were considered. Thirteen sequences, including one known gene and twelve pseudogenes were retrieved from different chromosomal regions and the size of sequences ranges from ~1,000 to ~2,700 bp. The percentages of N and G+C composition have been calculated (Table 5.5). If the Ns are included in sequences, the percentage of G+C composition ranges from 26.23% to 41.45%. If Ns are not counted in sequences, the percentage of G+C composition ranges from 33.78% to 44.62%.

³² Cytochrome is a class of hemoprotein. The major biological function is for electron transport by virtue of a reversible valency change of the heme iron.

Table 5.5: Percentage of N and G+C Composition in Sequences

estGroupID	length	N	pctN	G+C	pctG+C	
BC005299-1	2427	0	0.00%	1006	41.45%	(41.45%)
BC005299-2	1564	314	20.08%	442	28.26%	(35.36%)
BC005299-3	1219	0	0.00%	413	33.88%	(33.88%)
BC005299-4	1237	4	0.32%	425	34.36%	(34.47%)
BC005299-5	1210	0	0.00%	419	34.63%	(34.63%)
BC005299-6	1215	3	0.25%	413	33.99%	(34.08%)
BC005299-7	2686	436	16.23%	957	35.63%	(42.53%)
BC005299-8	1247	0	0.00%	433	34.72%	(34.72%)
BC005299-9	1216	0	0.00%	414	34.05%	(34.05%)
BC005299-10	1548	346	22.35%	406	26.23%	(33.78%)
BC005299-11	1784	233	13.06%	692	38.79%	(44.62%)
HUMCYCPS1-1	1060	0	0.00%	379	35.75%	(35.75%)
HUMCYCPS1-2	1366	310	22.69%	389	28.48%	(36.84%)

Column 1, estGroup; column 2, sequence length; column 3, number of Ns in sequence; column 4, percentage of Ns in sequence; column 5, number of G+C composition in sequence; column 6, percent of G+C composition in sequence; column 7, percentage of G+C composition (without Ns) in sequence.

The start codon (ATG) and its alternative patterns (Table 5.1) were reviewed and the number of occurrences was detected. All thirteen sequences (including forward and reverse strands) contain the standard start codon ATG (Table 5.6).

The stop codons (TGA, TAG and TAA) and their alternative patterns (Table 5.2) were reviewed. Among the standard stop codon patterns, the frequency of TAA is greater than other stop patterns in most cases. The number of counts for each estGroup is listed in Table 5.7.

Table 5.6: Frequency of Start Codon Patterns

estGroupID	ATG	ATN		ANG		ANN		NTG		NNG		NTN		NNN	score
	counts	# patterns	counts	# patterns	counts	# patterns	counts	# patterns	counts	# patterns	counts	# patterns	counts	counts	
BC005299-1F	30	3	136	3	107	9	399	3	103	8	156	9	399		4.8906
BC005299-1R	41	3	124	3	116	9	387	3	132	8	243	9	379		4.8906
BC005299-2F	26	3	86	3	57	11	225	3	57	9	83	9	201	104	5.0781
BC005299-2R	20	3	92	3	46	9	208	3	53	9	59	9	228	104	4.9531
BC005299-3F	17	3	98	2	41	9	202	3	59	7	55	9	218		4.5781
BC005299-3R	32	3	84	3	57	9	219	3	53	6	64	9	193		4.7656
BC005299-4F	32	3	82	3	54	10	226	3	58	7	72	9	178	1	4.8906
BC005299-4R	18	3	94	3	40	9	204	3	59	9	56	9	220	1	4.9531
BC005299-5F	18	3	85	3	37	9	207	3	58	8	56	9	223		4.8906
BC005299-5R	20	3	84	2	54	9	226	3	62	9	74	8	186		4.6406
BC005299-6F	19	3	87	3	44	10	222	3	58	7	49	9	216	1	4.8906
BC005299-6R	26	3	83	3	58	11	214	3	58	7	65	9	202	1	4.9531
BC005299-7F	48	3	109	3	102	10	375	3	117	9	195	9	328	144	5.0156
BC005299-7R	34	4	121	3	89	9	350	3	138	9	168	10	339	144	5.2656
BC005299-8F	23	3	87	3	54	9	222	3	62	8	78	9	197		4.8906
BC005299-8R	17	3	93	3	48	9	206	3	65	8	258	9	222		4.8906
BC005299-9F	23	3	90	3	41	9	210	3	55	5	57	9	216		4.7031
BC005299-9R	24	3	89	3	53	9	217	3	63	6	64	9	194		4.7656
BC005299-10F	20	3	91	3	42	9	204	3	49	7	55	10	222	114	4.8906
BC005299-10R	30	3	82	3	58	11	212	3	56	7	57	9	196	114	4.9531
BC005299-11F	37	3	76	3	103	9	240	3	66	8	155	10	180	77	4.9531
BC005299-11R	28	3	82	3	41	11	204	3	88	8	110	9	257	77	5.0156
HUMCYCPS1-1F	17	3	82	3	38	9	179	3	51	7	53	9	174		4.8281
HUMCYCPS1-1R	28	3	69	3	47	9	178	3	48	3	66	9	172		4.5781
HUMCYCPS1-2F	23	3	71	3	48	10	180	3	47	9	76	9	171	103	5.0156
HUMCYCPS1-2R	18	3	75	3	42	9	173	3	45	8	54	9	181	103	4.8906

F, forward strand; R, reverse strand; #, number. The patterns of start codon were listed in Table 5.1.

Formula to calculate score:

$$\text{score} = \text{ATG} \times 1.00 + \# \text{ of ATN patterns} \times 0.25 + \# \text{ of ANG patterns} \times 0.25 + \# \text{ of ANN patterns} \times 0.0625 + \# \text{ of NTG patterns} \times 0.25 \\ + \# \text{ of NNG patterns} \times 0.0625 + \# \text{ of NTN patterns} \times 0.0625 + \text{NNN} \times 0.015625$$

Table 5.7: Frequency of Stop Codon Patterns

estGroupID	TGA	TAG	TAA	TGN		TNG		TAN		TNA		TNN		NAA	
	counts	counts	counts	# patterns	counts	# patterns	counts	# patterns	counts	# patterns	counts	# patterns	counts	# patterns	counts
BC005299-1F	42	21	71	3	89	2	51	2	68	2	111	4	215	3	154
BC005299-1R	44	29	67	3	125	2	64	2	65	2	113	4	165	3	146
BC005299-2F	23	16	44	3	56	2	27	2	45	2	69	4	86	3	89
BC005299-2R	21	15	48	3	51	2	24	2	42	2	67	5	125	3	75
BC005299-3F	25	12	49	3	51	1	25	2	41	2	67	4	124	3	70
BC005299-3R	25	13	42	3	57	1	20	2	46	2	74	4	81	3	97
BC005299-4F	26	14	40	3	61	2	24	2	43	2	69	4	79	3	94
BC005299-4R	28	13	41	3	49	2	25	2	45	2	66	4	126	3	76
BC005299-5F	23	13	42	3	51	2	21	2	44	2	62	4	128	3	76
BC005299-5R	20	17	42	3	60	2	27	2	39	2	65	4	77	3	91
BC005299-6F	25	12	46	3	50	1	22	2	42	2	65	4	118	3	81
BC005299-6R	23	15	42	3	58	2	27	2	40	2	71	4	85	3	87
BC005299-7F	40	28	50	3	123	2	31	2	53	2	105	5	164	3	132
BC005299-7R	48	20	57	3	124	2	41	2	56	2	90	6	198	3	119
BC005299-8F	25	17	42	3	57	2	25	2	43	2	72	4	83	3	92
BC005299-8R	25	10	47	3	45	2	23	2	45	2	67	4	124	3	78
BC005299-9F	29	15	43	3	49	1	24	2	40	2	66	4	117	3	79
BC005299-9R	24	13	42	3	60	2	21	2	43	2	72	4	89	3	88
BC005299-10F	23	14	47	3	44	2	22	3	39	2	72	5	121	3	76
BC005299-10R	26	15	46	3	57	2	23	2	38	2	70	4	82	3	85
BC005299-11F	28	24	29	4	74	2	28	2	30	2	50	5	93	3	103
BC005299-11R	29	20	21	3	85	2	32	2	45	2	47	4	167	3	78
HUMCYCPS1-1F	24	9	40	3	44	1	19	2	33	2	54	4	101	3	66
HUMCYCPS1-1R	23	11	31	3	50	2	19	2	39	2	64	2	78	3	75
HUMCYCPS1-2F	21	13	33	3	46	2	23	2	38	3	57	4	76	3	129
HUMCYCPS1-2R	19	12	38	3	44	2	19	2	35	2	54	5	100	3	64

F, forward strand; R, reverse strand. The patterns of stop codon were listed in Table 5.2.

Table 5.7: Frequency of Stop Codon Patterns (Continued)

estGroupID	NAG		NGA		NNA		NNG		NAN		NGN		NNN counts	score
	# patterns	counts	# patterns	counts	# patterns	counts	# patterns	counts	# patterns	counts	# patterns	counts		
BC005299-1F	3	120	3	75	6	219	8	172	6	242	6	145		11.1406
BC005299-1R	3	144	3	120	5	138	8	225	6	216	6	171		11.0781
BC005299-2F	3	69	4	60	6	108	9	77	7	131	6	62	104	11.5156
BC005299-2R	3	57	3	38	6	117	9	61	6	133	6	61	104	11.4531
BC005299-3F	3	56	2	31	6	116	7	57	6	134	5	57		10.5156
BC005299-3R	3	66	2	52	6	104	7	63	6	128	5	55		10.5156
BC005299-4F	3	71	3	57	6	107	7	67	6	129	6	58	1	11.0781
BC005299-4R	3	53	3	27	6	118	9	62	6	135	5	59	1	11.1406
BC005299-5F	3	53	3	28	6	116	8	62	6	124	5	58		11.0781
BC005299-5R	3	68	3	58	6	108	7	75	6	127	6	55		11.0781
BC005299-6F	3	57	3	31	6	113	8	57	6	131	5	56	1	10.8281
BC005299-6R	3	70	2	53	6	104	7	64	7	127	6	56	1	10.8906
BC005299-7F	4	158	4	105	7	202	9	170	7	211	7	151	144	12.1406
BC005299-7R	3	132	4	76	6	204	9	179	6	218	6	138	144	11.9531
BC005299-8F	3	66	3	45	6	100	8	79	6	127	6	66		11.1406
BC005299-8R	3	58	3	30	6	117	8	64	6	131	6	65		11.1406
BC005299-9F	3	66	3	31	6	116	6	49	6	105	5	64		10.7031
BC005299-9R	3	66	3	73	5	84	6	69	6	132	5	52		10.8906
BC005299-10F	3	61	3	29	6	110	7	48	6	127	6	63	114	11.8281
BC005299-10R	3	66	3	57	7	98	7	64	7	132	6	39	114	11.2031
BC005299-11F	3	124	3	96	6	150	8	143	6	145	6	98	77	11.6406
BC005299-11R	3	62	3	42	7	128	8	114	7	133	7	88	77	11.3281
HUMCYCPS1-1F	3	50	3	31	6	100	8	58	6	122	6	49		10.8906
HUMCYCPS1-1R	3	61	2	45	6	86	8	58	6	107	6	56		10.3906
HUMCYCPS1-2F	2	34	2	16	6	91	9	67	7	107	6	57	103	11.2656
HUMCYCPS1-2R	3	51	3	35	6	97	8	57	6	112	6	53	103	11.3906

F, forward strand; R, reverse strand. The patterns of stop codon were listed in Table 5.2.

Formula to calculate score:

$$\begin{aligned}
 \text{score} = & \text{TGA} \times 1.00 + \text{TAG} \times 1.00 + \text{TAA} \times 1.00 + \# \text{ of TGN patterns} \times 0.25 + \# \text{ of TNG patterns} \times 0.50 \\
 & + \# \text{ of TAN patterns} \times 0.50 + \# \text{ of TNA patterns} \times 0.25 + \# \text{ of TNN patterns} \times 0.25 + \# \text{ of NAA patterns} \times 0.25 \\
 & + \# \text{ of NAG patterns} \times 0.25 + \# \text{ of NGA patterns} \times 0.25 + \# \text{ of NNA patterns} \times 0.0625 \\
 & + \# \text{ of NNG patterns} \times 0.0625 + \# \text{ of NAN patterns} \times 0.0625 + \# \text{ of NGN patterns} \times 0.0625 + \text{NNN} \times 0.015625
 \end{aligned}$$

Table 5.8: Frequency of Poly-A Signal Patterns

estGroupID	AATAAA	ATTA AA	AGTAAA	TATAAA	CATAAA	GATAAA	AATATA	AATACA	AATAGA	AAAAAG	ACTAAA	AAGAAA	AATGAA	TTTAAA	AAAACA	GGGGCT	score
BC005299-1F	2	3	2	2	2	2	2	3		3	1	3	3	9	2		6.80
BC005299-1R	1	7	1	1	1	1	2	1		3	2	2	1	9	3		7.05
BC005299-2F		4	1		1	1	1		1	3	2	2	1	6	1		3.95
BC005299-2R	2	2	1	1	2		2	2		1	1		1	6	1		4.75
BC005299-3F	3	2	1	2	2		2	1		1	1		2	7	1		5.95
BC005299-3R	3		1	1		1	2			3	2	2	1	7			5.00
BC005299-4F		3	1	1	2	1	1			4		1	1	5	1		3.25
BC005299-4R	1	1	1	4	1		3	2			1	5		5	2		3.80
BC005299-5F	2	1		4	2		3	1		1	1		1	5	1		4.35
BC005299-5R		2	1		1	1	1		1	3	2	2	1	5	1		2.85
BC005299-6F	2	1	1	3	2		4	2			1	1	2	6	1		4.75
BC005299-6R		3	1		1	1				2		1	1	6	2		2.90
BC005299-7F		3	1		1	1	1		1	2				4	1		2.65
BC005299-7R	1	1	1	4	3	4	2	2		3	1		2	4	1		4.15
BC005299-8F	1	1	1			2	2		1	2		2	1	7	1		3.35
BC005299-8R	2	2	1	5	1		3	2		1	1		2	7	1		5.35
BC005299-9F	1	3	2	3	1		3	1		1	1		1	5	1		4.35
BC005299-9R		2	1		2	1	2		1	3	1		2	5	1		2.85
BC005299-10F	1	2		5	2		2	2		1	1		2	5	2		4.10
BC005299-10R		3	1	1	1	1	1	1	1	3		1	1	5	1		3.25
BC005299-11F	3		1				1	1	2		1			2	3		3.95
BC005299-11R					1		4		2	1		1		1	1	1	1.10
HUMCYCPS1-1F	3	2	1	2	2		2	1		1	1		3	5	1		5.85
HUMCYCPS1-1R		2	1			1	1			2	1	1	1	5			2.30
HUMCYCPS1-2F		2	1				1		1	2	1	1	1	4	1		2.25
HUMCYCPS1-2R	2	2	1	1	2		2	1		1	1		1	4	1		4.45
	30	54	25	40	33	18	50	23	11	47	24	25	32	139	32	1	

Formula to calculate score:

$$\begin{aligned}
 \text{score} = & \# \text{ of AATAAA} \times 1.00 + \# \text{ of ATTA AA} \times 0.50 + \# \text{ of AGTAAA} \times 0.1 + \# \text{ of TATAAA} \times 0.1 + \# \text{ of GATAAA} \times 0.1 \\
 & + \# \text{ of AATATA} \times 0.1 + \# \text{ of AATACA} \times 0.1 + \# \text{ of AATAGA} \times 0.1 + \# \text{ of AAAAAG} \times 0.1 + \# \text{ of ACTAAA} \times 0.1 \\
 & + \# \text{ of AAGAAA} \times 0.1 + \# \text{ of AATGAA} \times 0.1 + \# \text{ of TTTAAA} \times 0.1 + \# \text{ of AAAACA} \times 0.05 + \# \text{ of GGGGCT} \times 0.05
 \end{aligned}$$

Table 5.9: Poly-A Site Information for Each Sequence

estGroupID	length	start codon location	stop codon	stop codon location	% finding poly-A site	strand direction	poly-A site	poly-A site location
BC005299-1	2427	1140	TAA	1556	59.25%	Reverse	ATTAAA	2137
BC005299-2	1564	68	TAA	698	38.18%	Forward	ATTAAA	1272
BC005299-3	1219	27	TAA	342	29.76%	Reverse	ATTAAA	919
BC005299-4	1237	64	TAA	382	30.20%	Forward	ATTAAA	1160
BC005299-5	1210	17	TAA	323	29.54%	Reverse	ATTAAA	1114
BC005299-6	1215	21	TTG	333	29.66%	Reverse	ATTAAA	1120
BC005299-8	1247	65	TAC	368	30.44%	Forward	AGTAAA	585
BC005299-9	1216	17	TAC	315	29.69%	Reverse	ATTAAA	1123
BC005299-10	1548	27	TAC	318	37.79%	Reverse	ATTAAA	1455
HUMCYCPS1-1	1060	76	TAA	391	25.88%	Reverse	ATTAAA	968
HUMCYCPS1-2	1366	70	TAA	700	33.35%	Forward	ATTAAA	1274

Sixteen patterns of poly-A signals were reviewed as well. The number of times each of the patterns was occurred were accumulated and listed in Table 5.8. In most cases, AATAAA, ATTAAA and AGTAAA were found. CATAAA was only found in BC005299-11 (marked in blue). In addition, that was the only sequence found the GGGGCT poly-A signal (marked in pink).

After running `blastx`, BC005299-7 and BC005299-11 were found to relate to other protein sequences. These two sequences were removed from pseudogene candidates. Based on the `blastx` results, the strand direction for each sequence was found. In addition, the start and stop codon locations for remain eleven sequences were recognized. Since the poly-A site is a six-base pattern, it has $4^6 = 4096$ combinations. The percentage of finding a poly-A signal is calculated (% of finding poly-A site = sequence length $\div$ 4096). With the information where the stop codons were located, the poly-A sites were found in the 3' UTR. It is surprising to find that ten out of eleven sequences have ATTAAA as the poly-A sites. Only BC005299-8 has AGTAAA as its poly-A signal. None of the sequences has found the most common poly-A signal AATAAA in the 3' UTR, using the stop codon location as a reference (Table 5.9). In addition, seven out of thirteen sequences have found TAA as stop codons. One has TTG and three have TAC as stop codons.

Based on the comparison results provided by `blastx`, the nucleotide sequences of each estGroup were retrieved. Each was compared to the cytochrome sequence at the nucleotide level in order to calculate the number of mutational events (similar synonymous, synonymous and nonsynonymous mutations) defined in Section 5.3.5. The similar synonymous mutation is marked with lower case 's'. The synonymous mutation is

marked with upper case ‘S’. The nonsynonymous mutation is marked with upper case ‘N’. In addition, if a gap was inserted in the `blastx` alignment result, either in the subject or query sequence, the number of gaps for each `estGroup` was also counted. If the insertion occurs in the subject sequence, it is marked with caret sign ‘^’. If the insertion occurs in the query sequence, it is marked with minus sign ‘-’ sign. If a premature stop codon was found in the query sequence, the nonsynonymous mutation was marked as lower case ‘p’. But if a premature stop codon was found in the query sequence and there is an insertion found in the subject sequence, the nonsynonymous mutation was marked as lower case ‘q’. In addition, the number of non-mutational nucleotide is marked as a blank ‘ ’. The results are shown in Table 5.10.

Table 5.10: Counts of Similar Synonymous Mutations, Synonymous Mutations, Nonsynonymous Mutations, and Insertion for Each Sequence

estGroupID	N	S	s	-	^	p	q		
BC005299-1	0	0	0	0	0	0	0	315	315
BC005299-2	7	3	2	0	0	0	0	303	315
BC005299-3	5	5	0	0	0	0	0	305	315
BC005299-4	11	10	5	0	0	0	0	289	315
BC005299-5	11	7	5	9	0	0	0	283	315
BC005299-6	18	6	5	3	0	2	0	281	315
BC005299-8	14	6	9	3	0	1	0	273	306
BC005299-9	15	5	12	0	0	0	0	262	294
BC005299-10	52	6	13	15	0	0	0	220	306
HUMCYCPS1-1	5	5	0	0	0	0	0	305	315
HUMCYCPS1-2	7	3	2	0	0	0	0	303	315

N, nonsynonymous mutation; S, synonymous mutation; s, similar synonymous mutation; -, insertion in query sequence; ^, insertion in subject sequence; p, nonsynonymous mutation when a stop codon appears in the query sequence; q, insertion occurs in the subject sequence when a stop codon occurs in the query sequence; blank ‘ ’, the number of non-mutational nucleotides; the last column represents the total number of nucleotides within the sequence.

In Section 5.3.3, a poly-A tail was defined as having a length of ten or more adenine (A) residues. However, the computational program did not return results for eleven candidate sequences. It is possible that none of the sequences have a poly-A tail. It is necessary to study the sequence file manually in order to check if the sequence does not have a poly-A tail in the 3' UTR. In fact, there are poly-A tails in the 3' UTR. However, the longest size of adenine (A) residues is seven bases. Since the program requires having ten adenines (A) as a minimum number, if a sequence pattern is like AAAAAAAGAAA, the program will not count it as a poly-A tail because the nucleotide G has broken the length. Therefore, eight out of eleven pseudogene candidate sequences have poly-A tails, after reviewing the sequence file. The size of poly-A tails ranges from eleven to 29 bases (Table 5.11).

A score was generated and each pseudogene factor was assigned a value as defined in Section 5.3. Table 5.11 provides the scoring results for each candidate pseudogene sequence. The unit score for each column is shown in the bottom of the table. The score for start codon, stop codon and poly-A sites have been defined in Tables 5.1 to 5.3. For poly-A tails, if the size is less than twenty adenines (A), each base is giving a 0.1. If the poly-A tail has more than twenty adenines (A), then 2.0 is given.

Table 5.11: Score for Pseudogene Candidate Sequences

estGroupID	start codon	stop codon	poly-A site	poly-A tails	N	S	s	-	^	p	q		score
BC005299-1	ATG	TAA	ATTAAA	0	0	0	0	0	0	0	0	315	5.65
BC005299-2	ATG	TAA	ATTAAA	15	7	3	2	0	0	0	0	303	7.86
BC005299-3	ATG	TAA	ATTAAA	23	5	5	0	0	0	0	0	305	8.10
BC005299-4	ATG	TAA	ATTAAA	15	11	10	5	0	0	0	0	289	8.34
BC005299-5	ATG	TAA	ATTAAA	13	11	7	5	9	0	0	0	283	8.95
BC005299-6	ATG	TTG	ATTAAA	11	18	6	5	3	0	2	0	281	8.27
BC005299-8	ATG	TAC	AGTAAA	26	14	6	9	3	0	1	0	273	8.64
BC005299-9	ATG	TAC	ATTAAA	29	15	5	12	0	0	0	0	262	8.77
BC005299-10	ATG	TAC	ATTAAA	29	52	6	13	15	0	0	0	220	13.61
HUMCYCPS1-1	ATG	TAA	ATTAAA	0	5	5	0	0	0	0	0	305	6.10
HUMCYCPS1-2	ATG	TAA	ATTAAA	0	7	3	2	0	0	0	0	303	6.36
unit score	Table 5.1	Table 5.2	Table 5.3	1.0~2.0	0.1	0.01	0.05	0.1	0.05	0.1	0.05	0.01	

N: nonsynonymous mutation; S: synonymous mutation; s: similar synonymous mutation; -: gap insertion in the query sequence; ^: gap insertion in the subject sequence; p: nonsynonymous mutation occurred to make the coding codon becomes stop codon in query sequence; q: gap insertion in the subject sequence because stop codon is found in query sequence.

Formula to calculate score: #, number

score = start codon + stop codon + poly-A site + score of poly-A tail (1.0~2.0) + # of N's × 0.1 + # of S's × 0.01 + # of s's × 0.05 + # of -'s × 0.1 + # of ^'s × 0.05 + # of p's × 0.1 + # of q's × 0.05 + # of blank ' ' × 0.01

5.7 Discussion

BC005299-1 was retrieved from chromosome 7. It is the only sequence that does not contain any mutation in the 315-base aligning to the cytochrome protein sequence, when running `blastx`. The `blastx` returned the alignment results in two pieces of 100% sequence identity, with a gap of 100 bases. It is most likely to be a gene itself. In fact, these 100 bases represent an intron.

BC005299-2 and HUMCYCPS1-2 are another two sequences whose `blastx` results return two pieces. Both of them were retrieved from chromosome 6. After reviewing the sequences themselves, the sequences in between the two segments are mainly Ns. The size ranges from ~50 to ~310 bases. In fact, both sequences were retrieved from overlapping regions in chromosome 6 with a ~1100- base overlap. BC005299-3 and HUMCYCPS1-1 yield similar `blastx` results. Both of them came from chromosome 8. The only difference is that BC005299-3 has a poly-A tail but HUMCYCPS1-1 does not. After reviewing the sequences in genome, they were found have a ~1000-base overlap. BC005299-9 which was retrieved from chromosome 2 is another sequence that has four bases inserted between two segments. Otherwise, the rest of five sequences are all in one piece. The size ranges from 291 to 315.

Besides BC005299-1, the sequence similarity for the rest of the sequences in Table 5.11 ranges from 63% to 95%. All of them contain poly-A signals. Eight out of the ten sequences have poly-A tails. BC005299-10 from chromosome 3 has been found the most number of nonsynonymous mutations. In addition, when compared to the cytochrome protein sequence, the sequence has to carry more gaps in order to receive the

best scoring results. BC005299-6 from chromosome 8 and BC005299-8 from chromosome 9 have premature stop codons.

The nonsynonymous mutations (marked as upper case 'N' in Table 5.11) are assigned a higher score (0.1) than the synonymous mutation (marked as upper case 'S' and lower case 's', scored 0.05) because of corresponding changes to the amino acid. Similar synonymous mutations have higher score (0.05) than the synonymous mutation (0.01). Although the amino acid is changed to a close genetic code, the amino acid is not the same one anymore. If the error occurs again when sequences are duplicated, the correct nucleotide(s) would not only be copied.

Insertions occurred in the query sequence in `blastx` result (marked as minus sign '-') are assigned a higher score (0.1) than the insertion in subject sequence (marked as caret sign '^', scored 0.05). Gap insertions are only for the computational purpose in order to calculate the best comparison result. In addition, when comparison made between the query and subject sequence, if gaps are inserted to the query sequence, there are a number of nucleotides deleted in the query sequence.

The premature stop codon which is found in the query sequence causes the nonsynonymous mutation (marked as lower case 'p'). It gets assigned a score of 0.1. If the premature stop codon is found in the query sequence, but the subject sequence is inserted a gap in order to get a better score in sequence comparison, this nonsynonymous mutation (marked as lower case 'q') has a score of 0.05.

The scores for start codons, stop codons and poly-A sites are given in Tables 5.1 to 5.3. After adding up single score for each sequence, the score ranges from 5.65 to 13.61. BC005299-1, the cytochrome gene itself has the lowest score 5.65. BC005299-10

has the highest score due to having the highest number of nonsynonymous mutations occurring in the sequence.

CHAPTER VI

CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

To understand gene regulation and expression, expressed sequence tags (ESTs) have been used widely in sequence alignment. This dissertation has discussed the implementation of using EST sequences for finding the efficiency of current BLAST tools, determining undetected alternative splicing events, and detecting and characterizing pseudogenes in genomic sequences in Chapters 3 to 5. These projects yield many interesting research directions to explore upon completion of doctoral work. A few of these are described in Sections 6.1 to 6.4.

6.1 BLAST Comparison Project

In the BLAST comparison project (Chapter 3), ESTs serve as short sequences as both query and database targets for sequence alignment. Tests were made under default settings for all three chosen BLAST tools. In general, without knowing the sequence length and file size, WU BLAST was found to be the best for sequence comparison at a nucleotide level. Results may vary when comparing amino acid sequences. The BLAST comparison project was completed in 2004. All three search tools have since released updated versions. New features have been added to provide better and accurate search results. There are additional versions, such as PSI-BLAST (101), PHI-BLAST (102) and megaBLAST (223) in ncbiBLAST, as well as other parallel implementations (224-227).

These were not tested, and may yield more efficient results. In addition, fine tuning the parameters may result in varying efficiencies.

6.2 Rat Phosphodiesterase 4 (PDE4) Project

In Chapter 4, computational detection of alternative splicing events was discussed. Different combinations of exons can produce different mRNA sequences. ESTs can provide partial information of the mRNA. Moreover, after high-throughput analysis was established, an increasing number of ESTs have been produced from different tissue types in the wet lab. In order to find alternative splicing variants, ESTs become a good resource. Phosphodiesterase 4 (PDE4) genes were chosen to study in the project in order to find the relationship between tissue specificity and isoforms. A computational method has been designed and implemented into programs to align rat ESTs to the corresponding chromosomal and mRNA sequences. In addition, the splice patterns and tissue type for each EST was examined to gain more insight into PDE4 isoforms tissue specificity.

Some undetected isoforms were found by the computational method. After using primers to sequence these patterns found in the PCR experiments, a number of novel isoforms were experimentally verified. Results are shown in Sections 4.8. Further collaboration with web lab biologists could result in confirmation of novel tissue specific isoforms. Additional primers can be designed and run in gels in order to verify more of the predicted patterns. These patterns could be further localized within tissues using *in*

situ hybridization³³. The complete sequence of the isoform is desired, and could be determined using techniques such as RACE sequencing³⁴.

The methodology for finding alternative splicing events in PDE4 has been designed and implemented in several scripts. In the future, this process could be automated, allowing users to input a gene of interest. The results provided will allow for users conduct wet lab experiments for confirmation of predicted isoforms.

This PDE4 project focused in rat genome. Future extensions could look at incorporation of EST information from comparative genomes (such as mouse, chimpanzee and human) for detection of rare splicing events.

6.3 Computational Method of Detecting Alternative Splicing Events

The method developed for finding splicing variants concentrates on finding functional alternative splices. Future studies could distinguish non-functional splices from functional ones. However, this would require more biological information on the difference between functional and non-functional alternative splicing events.

6.4 Computational Method for Pseudogene Detection and Characterization

In addition to finding alternative splicing sequences in genomic regions, ESTs were also used to determine pseudogenes in genome. Pseudogenes have been defined as

³³ *In situ* hybridization is a technique in which single-stranded DNA are permitted to interact so that hybrids are formed by molecules with sufficiently similar, complementary sequences. It involves hybridizing a labeled nucleic acid in fluorescent dye to suitably prepared cells or histological sections. *In situ* hybridization is used particularly to look for specific transcription or localization of genes to specific chromosomes.

³⁴ RACE, or rapid amplification of cDNA ends, is a technique used in molecular biology to obtain the partial sequence of an RNA transcript found within a cell. RACE begins through RT-PCR to produce a cDNA copy of a region of the RNA transcript. An unknown end portion of a transcript is copied using a known sequence from the center of the transcript. The copied region is bounded by the known sequence, and either the 5' or 3' end.

sequences which look like genes (having similar genomic features) but are not able to produce proteins as genes. Instead of using cDNA or mRNA sequences to find genes themselves in the genomic regions. ESTs can help to locate pseudogene sequences that have never been reported before. A computational method was developed to detect and characterize pseudogenes in Chapter 5. The biological factors for pseudogenes were evaluated, such as finding premature stop codons and calculating the number of synonymous and nonsynonymous mutations within sequences. A scoring scheme has been established in order to evaluate sequences or not. Based on the scoring scheme presented in Section 5.3, it would be easier for users to distinguish pseudogenes from gene sequences.

However, the developed computational method for pseudogene detection and characterization still requires manual review. Therefore, it is necessary to modify the detecting method to calculate the percentage of the adenine (A) in the sequences.

In pseudogene detection and characterization, ESTs are used to locate the candidate pseudogene locates in genomic regions. For better evaluation, having `blastx` results will help to locate the exact locations of start and stop codons in the candidate regions. `blastx` results also help to calculate the synonymous and nonsynonymous mutation with the sequences. Therefore, to detect and characterize pseudogenes, the known information from protein sequences is necessary. In addition, based on where the start codons of pseudogene candidate sequences are located, it would be necessary to retrieve either upstream or downstream sequence from the genomic sequences to further analyze for the presence of a TATA box and transcription factors.

The methodology discussed in Chapter 5 was developed step-by-step and implemented into computer programs, including data preprocessing, sequence alignment, clustering and pseudogene evaluation. To extend the research in pseudogene detection and characterization, a pipeline process is planned from data preprocessing all the way to evaluation so pseudogene analysis can be performed in an automated fashion. The pipeline could be implemented to accommodate multiple genomes. The bottleneck in this process is currently the time it takes for sequence comparison. Future research could look into parallel and distributed computing applications to speed up this process.

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APPENDIX I

ABBREVIATIONS

ASN.1 – abstract syntax notation one
BLOSUM – blocks of amino acid substitution matrix
bp – base pair
cAMP – cyclic adenosine monophosphate
cDNA – complementary DNA
CDS – coding sequence
cGMP – cyclic guanosine monophosphate
CNS – central nervous system
COPD – chronic obstructive pulmonary disease
CSHL – Cold Spring Harbor Laboratory
CPU – central process unit
dbEST - expressed sequence tags database
DDBJ – DNA DataBank of Japan
ddNTP – dideoxynucleotide triphosphate
DNA – deoxyribonucleic acid
ELSI – ethical, legal, and social issues
EMBL – European Molecular Biology Laboratory
ENCODE – ENCyclopedia of DNA Elements
EST – expressed sequence tag
GI – GenInfo Identifier
GMAP – genomic mapping and alignment program
HGP – Human Genome Project
HSP – high-scoring pair
INSDC – International Nucleotide Sequence Database Collaboration
IUB – International Union of Biochemistry
IUPAC – International Union of Pure and Applied Chemistry
MPI – message passing interface
mRNA – messenger RNA
miRNA – microRNA
NAS – National Academy of Science
NCBI – National Center for Biotechnology Information

NIH – National Institutes of Health
NLM – National Library of Medicine
ORF – open reading frame
PAM – point accepted mutation
PBS – portable batch system
PCR – polymerase chain reaction
PDE – phosphodiesterase
PDE4 – phosphodiesterase 4
PPi – pyrophosphate
PSSM – position specific scoring matrix
RACE – rapid amplification of cDNA ends
RefSeq – Reference Sequence
RNA – ribonucleic acid
RNAi – RNA interference
rRNA – ribosomal RNA
SARS – severe acute respiratory syndrome
siRNA – silencing RNA, short interfering RNA or small interfering RNA
snRNA - small nuclear RNA
tRNA – transfer RNA
TIGR – The Institute for Genomic Research
UCSC – University of California Santa Cruz
UCR - upstream conserved region
UTR – untranslated region
WU - Washington University at Saint Louis
XML – extensible markup language

APPENDIX II

GLOSSARY

3' and 5'– Directionality in molecular biology refers to the end-to-end chemical orientation of a single strand of nucleic acid. The chemical convention of naming carbon atoms in the nucleotide sugar-ring numerically gives rise to a 5' end and a 3' end (usually pronounce “five prime end” and “three prime end”). Therefore, the 5' end designates the end of the DNA or RNA strand that has the fifth carbon in the sugar-ring of the (deoxy)ribose at its terminus. The 3' end has the third carbon in the sugar-ring as its terminus (Figure 1.1).

ab initio – In computation, *ab initio* means an extra, transform, load tool used to manipulate data. In Bioinformatics, *ab initio* is a term used to define methods for making predictions about biological features using only a computational model without extrinsic comparison to existing data. In this context, it may be sometimes interchangeable with the Latin term *de novo*.

alternative splicing – process of mRNA transcription through which the coding regions for a eukaryotic gene, called exons, are extracting and connected to produce alternative mRNA molecules.

amino acid – the basic elements of proteins. Amino acids form short polymer chains called peptides or longer sequences called polypeptides.

BLAST – stands for basic local alignment search tool which is widely used in Bioinformatics and Computational Biology to find out the basic alignment results between two sequences.

BLOSUM – stands for blocks of amino acid substitution matrix. It is a substitution matrix used for sequence alignment of proteins. It was generated by Henikoff and Henikoff in 1992. The most commonly used matrix is BLOSUM62.

cDNA – stands for complementary DNA. It is a single-stranded DNA made in a laboratory so that its base sequence is complementary to a messenger RNA template. It is assembled by the enzyme reverse transcriptase and may be used in gene cloning or as a gene probe.

central dogma – a process to derive a genetic DNA sequence to a fully-functional protein that contains transcription, pre-mRNA processing, translation and protein folding.

cleavage – a series of mitotic cell divisions that produces a blastula from a fertilized ovum. It is the basis of the multi-cellularity of complex organisms. It can be called segmentation as well.

cleavage site – a DNA sequence that can be recognized and cut by a specific restriction enzyme.

codon – a sequence of three adjacent nucleotides constituting the genetic code that determines the insertion of a specific amino acid in a polypeptide chain during protein synthesis or the signal to stop protein synthesis.

contig – a continuous sequence of DNA created by assembly overlapping sequenced fragments of a chromosome. It is also a group of clones representing overlapping regions of a genome.

CpG islands – short genomic regions that contain a high frequency of CG dinucleotides than in other regions. ‘p’ indicates that ‘C’ and ‘G’ are connected by phosphodiesterase bond.

cytochrome – a class of hemoprotein. The major biological function is for electron transport by virtue of a reversible valency change of the heme iron.

dbEST –a division of GenBank that collects ESTs submitted from around the world. It contains sequence data and its annotation information from numerous organisms.

ddNTP – stands for dideoxynucleotide, represents one of the four portions: ddATP, ddCTP, ddGTP and ddTTP.

de novo – anew, afresh, the same as if it had not been heard before and as if no decision previously had been rendered.

DNA – stands for deoxyribonucleic acid. It carries the genetic information in the cell and is capable of self-replication and synthesis of RNA. It has four bases –adenine (A), cytosine (C), guanine (G) and thymine (T). DNA consists of two long chains of nucleotides twisted into a double helix and joined by hydrogen bonds between the complementary bases A to T or C to G. The sequence of nucleotides determines individual hereditary characteristics.

DNA sequencing – a method to determine the order of the nucleotide bases, A, C, G and T, in a DNA oligonucleotide.

DUST – is a computational program used for masking or filtering low complexity regions in nucleic acids.

dynamic programming – a method of solving problems when the solutions can be determined recursively by building upon known solutions of a subspace.

electrophoresis – a method of separating substances, especially proteins, and analyzing molecular structure based on the rate of movement of each component in colloidal suspension while under the influence of an electric field. This method was introduced by Arne Tiselius in 1937 for separating proteins in solution.

ENCODE – stands for encyclopedia of DNA elements. It is an international-wide research to identify the functional elements in the human genome. ENCODE project contains three phase, a pilot phase, a technology development phase and a production phase.

EST – stands for expressed sequence tag, which is a segment of cDNA. The size ranges from 300 to 600 base pair. They are used to identify gene transcripts and are instrumental in gene discovery and gene sequence determination.

exon – is coding region in the genomic sequence. It is a discontinuous sequence of DNA that codes for protein synthesis and carries the genetic code for the final messenger RNA molecule.

e-value – is the sum of all possible values for a random variable, each value multiplied by its probability of occurrence. It is also the integral of the probability density function and a continuous random variable over its range of values.

FASTA – stands for FAST-ALL. It was developed by Pearson and Lipman. The method combines local search with a heuristic algorithm to search a database for sequence

homology. FASTA compares protein or nucleotide sequences to report the best matches of sequences for local alignment.

FASTA format – the data format begins with a greater-than symbol '>', followed by single line description. The next few lines are sequence data, the combination of A, C, G and T nucleic acids or twenty different amino acids.

frame shifting – a genetic mutation that inserts or deletes a number of nucleotides that is not evenly divisible by three.

GC% - the percentage of total number of G's and C's in the sequence.

GenBank – is a sequence data bank which was established in 1982 by the National Institutes of Health to help advance scientific discovery worldwide. GenBank collects DNA and RNA sequences and exchanges data to European Molecular Biology Laboratory and the DNA Data Bank of Japan daily.

GenBank format – the data format contains complete sequence information including locus, definition, accession, version, keywords, source, reference, comment, features, base count, and origin.

gene – the basic unit to transfer hereditary information in genome. Genes provide structure, function and regulation to a biological system.

GI – stands for GenInfo Identifier which is used as a unique sequence identifier for nucleotide and proteins. The GI number has been used by NCBI to track sequence histories in GenBank and the other sequences databases it maintains. The VERSION system of identifiers was adopted in 1999 by the International Nucleotide Sequence Database Collaboration (GenBank, EMBL and DDBJ).

genome annotation – a process of assigning biological information to sequences. The process is based on two main steps: identifying elements on the genome and adding the biological information to the elements. An annotation is a note added by way of commentary and explanation. Once a genome is sequenced, it needs to be annotated and clarified.

global alignment – is aligning the entire length of two sequences to find out sequence similarity.

Hamming distance – if two sequences of length N differ from each other at n sites, then the proportion of differences, n/N , is referred to as the Hamming distance or the degree of divergence.

heuristic algorithm – an algorithm that can make an approximation of the best solution without exhaustively considering every possible outcome. It tries to reduce time and space complexity in calculation.

high-throughput sequencing - a fast method of determining the order of bases in DNA.

homology – similar characteristics in two animals that are a product of descent from a common ancestor rather than a product of a similar environment.

Human Genome Project – a project was to sequence the human genome. The project began in 1990 to determine genes in the human genome. In 2001, the first draft was published. In 2003, 95% of the gene-containing part of the human sequence was mapped to 99.99% accuracy.

in silico – an expression used to mean performing on computer or via computer simulation.

***in situ* hybridization** – a technique in which single-stranded DNA are permitted to interact so that hybrids are formed by molecules with sufficiently similar, complementary sequences. It involves hybridizing a labeled nucleic acid in fluorescent dye to suitably prepared cells or histological sections. *In situ* hybridization is used particularly to look for specific transcription or localization of genes to specific chromosomes.

in vitro – a test performing in the glass or plastic vessels in the laboratory

intron – non-coding region in the genomic sequence. Introns are sections of DNA that are not expressed in the gene product.

isoform – a version of protein with only small differences to another isoform of the same protein. Different forms of a protein may be produced from different by related genes. It may also arise from the same gene by alternative splicing.

IUB – stands for International Union of Biochemistry. It provides recommendations on biochemical and organic nomenclature, symbols and terminology.

IUPAC – stands for International Union of Pure and Applied Chemistry. It serves to advance the worldwide aspects of the chemical sciences and to contribute to the application of chemistry in the service of Mankind.

local alignment – an algorithmic technique that looks for significant similarities in smaller sections of the sequences.

locus – the position that a given gene occupies on a chromosome .

low complexity region – the region of biased composition including homopolymeric runs, short-period repeats, and more subtle overrepresentation of one or a few residues.

MALDI-TOF-MS – stands for matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

MPI – stands for message passing interface which is a library specification for message passing. It is a standard application programming interface (API) that can be used to create parallel applications.

mpiBLAST – a parallel implementation using message passing interface to conduct NCBI BLAST in multi-processor cluster.

mRNA – stands for messenger RNA. It is the form of RNA that mediates the transfer of genetic information from the cell nucleus to ribosomes in the cytoplasm, where it serves as a template for protein synthesis. mRNA is synthesized from a DNA template during the process of transcription.

miRNA – stands for microRNA which are single-stranded RNA molecules about 21-23 nucleotides in length and regulates gene expression.

mutation – a change of the DNA sequence within a gene or chromosome of an organism resulting in the creation of a new character or trait not found in the parental type. Mutation can also mean the process by which such a change occurs in a chromosome, either through an alternation in the nucleotide sequence of the DNA coding for a gene or through a change in the physical arrangement of a chromosome.

NCBI – stands for National Center for Biotechnology Information. It is established in 1988 as a national resource for molecular biology information. NCBI creates public databases, conducts research in computational biology, develops software tools for analyzing genome data and disseminates biomedical information – all for the better understanding of molecular processes affecting human health and disease.

NIH – stands for National Institute of Health, which is the nation's medical research agency. NIH provides resources and financial support for scholars to conduct researches in medical related science.

nonsynonymous mutation – a mutation that occurs in the nucleotide level but affects the amino acid in translation.

oligonucleotide (or **oligo**) – a short polymer segment of RNA or DNA, the length ranges from two to twenty bases.

orthologous locus – genes that have evolved directly from an ancestral gene.

PAM – shorts for point accepted mutation. It is a matrix of score to consider the replacement of one amino acid in a protein by another residue that has been accepted by natural selection. It was released by Dayhoff and her colleagues in 1970s. The common used matrix for amino acid sequence comparison is PAM250.

PBS – stands for portable batch system. It is a scheduling system to conduct processes running on multi-processors in the Unix/Linux system. PBS provides additional controls over initiating or scheduling execution of batch jobs and allows routing of those jobs between different hosts.

PCR – stands for polymerase chain reaction. It is a method to amplify specific DNA segments by exploiting certain features of DNA replication *in vitro*. The PCR technique was developed by Kary Mullis in 1983. This technique uses a pair of primers, which are DNA segments approximately 20 bp long complement the DNA being synthesized and a thermo stable DNA polymerase to achieve near-exponential enzymatic amplification of target DNA.

PDE – stands for phosphodiesterase. PDEs are enzymes which act as regulators for signal transduction. Currently, there are eleven known families of PDEs. PDEs degrade cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP).

PDE4 – stands for phosphodiesterase 4 which is an enzyme being targeted for inflammatory disorders, including asthma and chronic obstructive pulmonary disease and central nervous system injury. PDE4 has a large number of potential isoforms with apparent tissue and functional specificity.

Pfam – Pfam (<http://pfam.sanger.ac.uk>) is a large collection of multiple sequence alignments and hidden Markov models covering many common protein domains and families. For each Pfam family, users are available to check for multiple alignments, view protein domain architectures, examines species distribution, connect to other databases, and study known protein structures.

polyadenyl (polyA) signal – a six-base sequence which appears in the 3' UTR. AATAAA is the most found pattern in mammalian genomes.

polyadenyl (polyA) tail – a sequence of 50 to 250 adenine (A) residues that are added to the 3' end of an mRNA sequence after being cleaved. This reaction is catalyzed by polyadenylate polymerase.

polymerase – a polymerase is an enzyme that can synthesize new DNA strands using a DNA template while several such enzymes exist.

primer – a segment of DNA or RNA that is complementary to a given DNA sequence and that is needed to initiate replication by DNA polymerase.

probe – a single-stranded DNA molecule used in laboratory experiments to detect the presence of complementary sequence among a mixture of other single-stranded DNA molecules.

promoter – a sequence of DNA that specifies the sites of transcription initiation for an RNA polymerase.

protein – a linear polymer built from amino acids. Proteins carry out all of a cell's activities.

pseudogene – a genomic sequence that looks like gene but does not have any functionality as gene. Currently there are three known types of pseudogenes: processed pseudogene, unprocessed pseudogene and disabled pseudogene.

RACE – short for rapid amplification of cDNA ends, is a technique used in molecular biology to obtain the partial sequence of an RNA transcript found within a cell. RACE begins through RT-PCR to produce a cDNA copy of a region of the RNA transcript. An unknown end portion of a transcript is copied using a known sequence from the center of the transcript. The copied region is bounded by the known sequence, and either the 5' or 3' end.

RefSeq – Reference Sequence database is a collection of annotated DNA, RNA and protein sequences from major organisms, including plasmids, organelles, viruses, archaea, bacteria and eukaryotes. RefSeq contains data extracted from GenBank and involves varying levels of computational and experimental verification from authoritative groups.

repetitive element – a sequence that appears more than once in a genome.

RepeatMasker – a software package designed to screen DNA sequences for repeats.

retrotransposition – a process occurring in the intermediate RNA stage that a transposon is created by reverse transcription of an RNA molecule.

ribosome – a minute round particle composed of RNA and protein. It is found in the cytoplasm of living cells and serves as the site of assembly by polypeptides encoded by messenger RNA.

RNA – stands for ribonucleic acid. It is a polymeric constituent of all living cells and many viruses. RNA consists of a long and single-stranded chain of alternative phosphate and ribose units with the bases adenine (A), cytosine (C), guanine (G), and uracil (U) bonded to the ribose. The structure and bases sequence of RNA are determinants of protein synthesis and the transmission of genetic information.

RNAi – stands for RNA interference. It is a specific, potent and highly successful approach for loss-of-functional studies in eukaryotic organisms. The process by which foreign, double-stranded RNA is recognized and degraded by specialized protein complex within many eukaryotic cells. It is believed to be an evolutionarily conserved defense mechanism against RNA viruses and transposable elements.

rRNA – stands for ribosomal RNA, which is a permanent structural part of a ribosome.

RT-PCR – reverse transcriptase polymerase chain reaction. It is a highly sensitive technique for the detection and quantization of mRNA. This technique includes two parts: the synthesis of cDNA from mRNA by reverse transcript and the amplification of a specific cDNA by the polymerase chain reaction.

SARS – stands for severe acute respiratory syndrome, is a severe form of pneumonia which appeared in outbreaks in 2003. It is transmitted especially by contact with infectious material (as respiratory droplets or body fluids), and is characterized by fever, headache, body aches, a dry cough, hypoxia and usually pneumonia.

SEG – a computational program for masking or filtering low-complexity regions in amino acids.

siRNA – stands for silencing RNA, short interfering RNA or small interfering RNA. siRNA results in high levels of knockdown for transient knockdown experiments.

Smith-Waterman Algorithm – is a well-known algorithm for performing local sequence alignment in order to determine similar regions between two nucleotide or protein sequences. Instead of looking at the total sequence, the Smith-Waterman algorithm compares segments of all possible length and optimizes the similarity measure.

snRNA – stands for small nuclear RNA and is a small RNA molecule that functions in the nucleus by guiding the assembly of macromolecular complexes on the target RNA to allow site-specific modifications or processing reactions to occur.

spliceosome – a nucleoprotein particle that aids in the splicing of messenger RNA in eukaryotes.

splice variant – an active mRNA that results from cutting and resealing by precise breakage of phosphodiester bonds at 5' and 3' splice sites (exon/intron junction).

It also indicates a recombinant DNA molecule derived from cutting and resealing of DNA from different sources.

splice site – located in either 5' or 3' ends of the intron sequence that spliceosome complex will bind on the sequence to conduct the splicing events in order to get the coding region sequences united. It is also called exon/intron junction. The most common splice site in mammalian genomes is GT/AG.

start codon – the initiating signal of translation and the first amino acid in a polypeptide chain.

stop codon – the terminating signal in genomic sequence to notify the gene to stop translation. There are three common stop codons: TGA, TAG, and TAA.

synonymous mutation – a mutation that occurs in the nucleotide level but does not affects the amino acid in translation.

synteny – the occurrence of two or more genes on the same chromosome, whether or not they are linked.

thread – a portion of a program that can run independently of and concurrently with other portions of the program.

TIGR – short for The Institute for Genomic Research. It was a non-profit genomics research institute founded in 1992 by Craig Venter in Rockville, Maryland. It is now part of the J. Craig Venter Institute (<http://www.tigr.org>).

transcription – the process that messenger RNA is synthesized from a DNA template resulting in the transfer of genetic information from the DNA molecule to the messenger RNA.

translation – the process by which messenger RNA directs the sequence of amino acids assembled by a ribosome during protein synthesis.

tRNA – stands for transfer RNA which is one of a class of RNA molecules that transport amino acids to ribosomes for incorporation into a polypeptide undergoing synthesis.

UCR – stands for upstream conserved region which are the regions being protected from harm or decay.

UniGene – an organized view of the transcriptome. UniGene provides sequence information in clusters that each UniGene entry is a set of transcript sequences that appear to come from the same transcription locus (gene or expressed pseudogene), together with information on protein similarities, gene expression, cDNA clone reagents and genomic location.

UTR – stands for untranslated region. It is a non-coding RNA that does not translated into a protein. It is located either in the 5' end before the start codon of a gene or 3' end after the stop codon is recognized in gene regulation.

XNU – a computational program for masking or filtering low-complexity regions in amino acids.

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PUBLICATIONS

Refereed Articles:

I. E. Cha, C. M. Whitaker, X. Li, N. G. F. Cooper and E. C. Rouchka, "Detection of Novel Rat PDE4 Isoforms Using Computational Prediction and Experimental Verification", in preparation.

I. E. Cha, K. I. Hoblitzell and E. C. Rouchka, "Alternative Splicing Events", University of Louisville Bioinformatics Technical Report Series, TR-ULBL-2007-03 (2007).

C. T. Chen, I. Cha and S. J. Hsieh, "Thermal Stability of Robust Unsymmetrical Copperporphyrins with Multiple Diphenyl and Nitro Substituents", *Journal of the Chinese Chemical Society*, 45: 741-748 (1998).

Conference Proceedings:

I. E. Cha and E. C. Rouchka, "Comparison of Current BLAST Software on Nucleotide Sequences", 4th International Workshop on High Performance Computational Biology (HiCOMB 2005), 19th International Parallel & Distributed Symposium Conference (IPDPS 2005), Denver, Colorado, April 4-8, 2005, DOI: 10.1109/IPDPS.2005.145.

Poster Presentations:

I. E. Cha, C. M. Whitaker, E. C. Rouchka, X. Li and N. G. F. Cooper, "Computational Method for Splicing Pattern Recognition in Rat PDE4 Sequences", KRBIN Semi-Annual Review, December 4, 2007

I. E. Cha, C. M. Whitaker, E. C. Rouchka, X. Li and N. G. F. Cooper, "Computational Method for Splicing Pattern Recognition in Rat PDE4 Sequences", UT-ORNL-KY Bioinformatics Summit 2007, April 14, 2007.

- I. E. Cha, C. M. Whitaker, E. C. Rouchka, X. Li and N. G. F. Cooper, “Computational Method for Splicing Pattern Recognition in Rat PDE4 Sequences”, National Engineering Week 2007, eExpo07, University of Louisville, March 3, 2007.
- I. E. Cha, C. M. Whitaker, E. C. Rouchka, X. Li and N. G. F. Cooper, “Automated Method for Splicing Pattern Recognition in Rat PDE4 Sequences”, Poster GRD20, Research! Louisville 2006, October 10, 2006.
- I. E. Cha, Y. Rudraraju, E. P. Stutzenberger and V. Akkineni, “The Sequence Alignment Problem – How to Get a Ligament from Alignment”, National Engineering Week 2006, eExpo06, University of Louisville, March 4, 2006.
- I. E. Cha and E. C. Rouchka, “Comparison of Current BLAST Software on Nucleotide Sequences”, Poster D-7, ISMB 2005, June 26, 2006.
- I. E. Cha and E. C. Rouchka, “Comparison of Current BLAST Software on Nucleotide Sequences”, UT-ORNL-KBRIN Bioinformatics Summit 2005, April 2, 2005.
- I. E. Cha and E. C. Rouchka, “Comparison of Current BLAST Software on Nucleotide Sequences”, National Engineering Days 2005, University of Louisville, February 20-21, 2005.
- E. C. Rouchka and I. E. Cha, “Bioinformatics”, National Engineers Days 2005, University of Louisville, February 20-21, 2005.
- E. C. Rouchka and I. E. Cha, “Bioinformatics”, National Engineers Days 2004, University of Louisville, March 26-27, 2004.

Invited Presentations:

- I. E. Cha, “*In silico* Prediction of Novel Tissue Specific Isoforms”, Center for Computational Biology and Bioinformatics, Indiana University – Purdue University Indianapolis, Indianapolis, Indiana, May 9, 2008.
(Dr. Sean Mooney, Host)
- I. E. Cha, “Computational Method for Recognition of Alternative Splicing”, Genomic Research Center, Academia Sinica, Taipei, Taiwan, R.O.C., August 24, 2007.
(Dr. C. Y. Lin, Host)
- I. E. Cha, “Computational Method for Splice Pattern Recognition in Rat PDE4 Sequences”, Section of Computer and Information Science, Kentucky Academy of Science (KAS 2006), Morehead University, Morehead, Kentucky, November 10, 2006.
- I. E. Cha, “Pseudogene Detection and Characterization”, Genomic Research Center, Academia Sinica, Taipei, Taiwan, R.O.C., December 23, 2005.
(Dr. C. Y. Lin, Host)

I. E. Cha, “Comparison of Current BLAST Software on Nucleotide Sequences”,
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PROFESSIONAL ORGANIZATIONS & SOCIETIES

Association of Computing Machinery (ACM)
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HONORS & AWARDS

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First Place Graduate Research Poster Competition Award, National Engineering Week
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First Place Graduate Research Competition Award, Section of Computer and Information
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Future Professor Program, University of Louisville, August 2003 – May 2004.