

Gene Finding

Strategies to find gene structures on the web

Swiss Institute of Bioinformatics (SIB)

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Introduction

Gene finding is about detecting coding regions and infer gene structure

Gene finding is difficult

- DNA sequence signals have low information content (degenerated and highly unspecific)
- It is difficult to discriminate real signals
- Sequencing errors

Prokaryotes

- High gene density and simple gene structure
- Short genes have little information
- Overlapping genes

Eukaryotes

- Low gene density and complex gene structure
- Alternative splicing
- Pseudo-genes
- ...

Gene finding strategies

Homology method

- Gene structure can be deduced by homology
- Requires a not too distant homologous sequence

Ab initio method

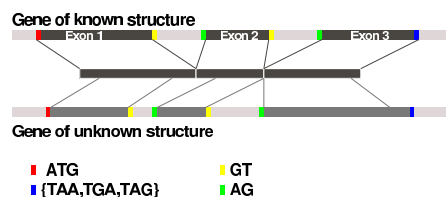
- Requires two types of information
 - ▷ compositional information
 - ▷ signal information

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Homology method

Principles of the homology method

- Coding regions evolve slower than non-coding regions, i.e. local sequence similarity can be used as a gene finder
- Homologous sequences reflect a common evolutionary origin and possibly a common gene structure, i.e. gene structure can be solved by homology (mRNAs, ESTs, proteins, domains).
- Standard homology search methods can be used (BLAST, Smith-Waterman, ...)
- Include "gene syntax" information (start/stop codons, ...)



Homology methods are also useful to confirm predictions inferred by other methods

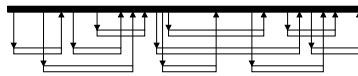
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Homology method (2)

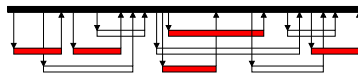
Procrustes is a software to predict gene structure from homology found in proteins (Gelfand *et al.*, 1996)

- Principle of the algorithm
 - ▷ Find all possible blocks (exons) in the query sequence (based on the acceptor/donor sites)
 - ▷ Find optimal alignments between blocks and model sequences
 - ▷ Find the best alignment between concatenation of the blocks and the target sequence

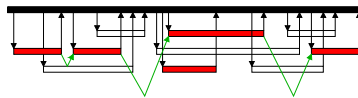
Find all possible true exons



Find homologous regions to a template protein



Find the best path



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Homology method (3)

Advantages of the homology method

- Successfully recognizes short exons and exons with unusual codon usage
- Assembles correctly complex genes (> 10 exons)
- Available on the web <http://www-hto.usc.edu/software/procrustes/qpn.html>

Problems of the homology method

- Genes without homologous in the databases are missed
- Requires close homologous to deduce gene structure
- Very sensitive to frame shift errors

Protocol to find gene structure using protein homology

- Do a BLASTX of your query sequence against a protein database (SWISS-PROT/TrEMBL)
- Retrieve sequences giving the best results
- Find gene structure using the retrieved sequences from the BLASTX search (Procrustes)
- BLAST the predicted protein against a protein database to verify the predicted gene structure

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Homology method (4)

Genewise uses HMMs to compare DNA sequences at the level of its conceptual translation, regardless of sequencing errors and introns

Principle

- The gene model used in genewise is a HMM with 3 base states (match, insert, delete) with the addition of more transition between states to consider frameshifts.
- Intron states have been added to the base model.
- Genewise directly compare HMM-profiles of proteins or domains to the gene structure HMM model.

Genewise can be used with the whole Pfam protein domain databases (find protein domain signatures in the DNA sequence)

Genewise is a powerful tool, but time consuming

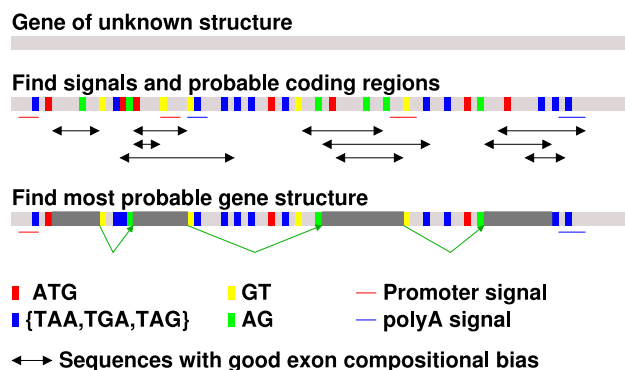
Genewise is part of the Wise2 package: <http://www.sanger.ac.uk/Software/Wise2>

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Ab initio method

Principles of the ab initio methods

- Integration of **signal detection** and **coding statistics**
- **Signal detection** and **coding statistics** are deduced from a training set
- Probabilistic frameworks are used to infer a probable gene structure
- A solid scoring system can be used to evaluate the predictions



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Signal detection

Detect short DNA motifs (promoters, start/stop codons, splice sites,...)

A number of methods are used for signal detection

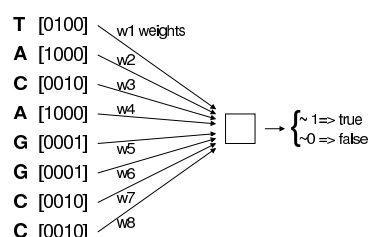
- Consensus string
 - ▷ Based on most frequently observed residues at a given position
- Pattern recognition
 - ▷ Flexible consensus strings
- Weight matrices
 - ▷ Based on observed frequencies of residues at a given position. Uses standard alignment algorithms. This method returns a score.
- Weight array matrices
 - ▷ Weight matrices based on dinucleotides frequencies. Takes into account the non-independence of adjacent positions in the sites.
- Maximal dependence decomposition (MDD)
 - ▷ MDD generates a model which captures significant dependencies between non-adjacent as well as adjacent positions, starting from an aligned set of signals.

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Signal detection (2)

Methods for signal detection (continuation)

- Hidden Markov Models (HMM)
 - ▷ HMM uses a probabilistic framework to infer the probability that a sequence correspond to a real signal
- Neural Networks (NN)
 - ▷ NN are trained with positive and negatives example and "discover" the features that distinguish the two sets.
Example: NN for acceptor sites, the *perceptron*, (Horton and Kanehisa, 1992)



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Signal detection (3)

Signal detection problem

- DNA sequence signals have low information content
- Signals are highly unspecific and degenerated
- Difficult to distinguish between true and false positive

How improve signal detection

- Take context into consideration (ex. acceptor site must be flanked by an intron and an exon)
- Combine with coding statistics (compositional bias)

Coding statistics

Inter-genic regions, introns, exons, ... have different nucleotides contents

This compositional differences can be used to infer gene structure

Examples of coding region finding methods:

- ORF length
 - ▷ Assuming an uniform random distribution, stop codons are present every 64/3 codons (≈ 21 codons) in average
 - ▷ In coding regions stop codon average decrease
 - ▷ Method sensitive to frame shift errors
 - ▷ Can't detect short coding regions
- Bias in nucleotide content in coding regions
 - ▷ Generally coding regions are G+C rich
 - ▷ There are exceptions. For example coding regions of *P. falciparum* are A+T rich
- Periodicity
 - ▷ Plot of the number of nucleotides separating pairs of T is periodic in coding regions, but not in non-coding regions

Coding statistics (2)

- Codon frequencies
 - ▷ Synonym codon usage is biased in a species dependent way
 - ▷ 3rd codon position: 90% are A/T; 10% are G/C
- How to calculate codon frequencies

Assume $S = a_1b_1c_1, a_2b_2c_2, \dots, a_{n+1}b_{n+1}c_{n+1}$ is a coding sequence with unknown reading frame. Let f_{abc} denote the appearance frequency of codon abc in a coding sequence. The probabilities p_1, p_2, p_3 of observing the sequence of n codons in the 1st, 2nd and 3rd frame respectively are:

$$\begin{aligned} p_1 &= f_{a_1b_1c_1} \times f_{a_2b_2c_2} \times \dots \times f_{a_nb_nc_n} \\ p_2 &= f_{b_1c_1a_2} \times f_{b_2c_2a_3} \times \dots \times f_{b_nc_n a_{n+1}} \\ p_3 &= f_{c_1a_2b_2} \times f_{c_2a_3b_3} \times \dots \times f_{c_n a_{n+1} b_{n+1}} \end{aligned}$$

The probability P_i of the i th reading frame for being the coding region is:

$$P_i = \frac{p_i}{p_1 + p_2 + p_3}$$

where $i \in \{1, 2, 3\}$.

Codon frequencies (2)

In practice we use these computations in a search algorithm as follows:

- Select a window of size n (for example $n = 30$)
- Slide the window along the sequence and calculate P_i for each start position of the window

A variation of the codon frequency method is to use 6-tuple frequencies instead of 3-tuple (codon) frequencies. This method was found to be the best single property to predict whether a window of vertebrate genomic sequence was coding or non-coding (*Claverie and Bougueleret, 1986*).

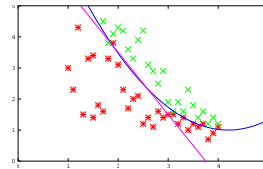
The usage of hexamers frequencies has been integrated in a number of gene predictors.

Integrating signal information and compositional information for gene structure prediction

A number of methods exists for gene structure prediction which integrate different techniques to detect signals (splicing sites, promoters, etc.) and coding statistics.

A non exhaustive list of the available methods:

- Linear and quadratic discrimination analysis
 - ▷ Linear discrimination analysis is a standard technique in multivariate analysis
 - ▷ Linear discrimination analysis is used to linearly combine several measures in order to perform the best discrimination between coding and non-coding sequences.
 - ▷ Quadratic discriminant analysis. Similar to linear discrimination analysis, but uses a quadratic discriminant function
 - ▷ Dynamic programming is used in to combine the inferred exons

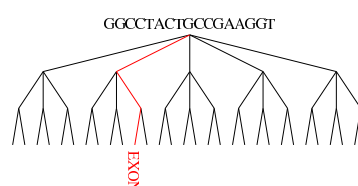


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Integrating signal information and compositional information for gene structure prediction (2)

A non exhaustive list of the available methods (continuation):

- Decision tree
 - ▷ Internal nodes of a decision tree are property values tested for each subsequence passed to the tree
 - ▷ Properties can be various coding measures (e.g. hexamer frequencies) or signal strengths
 - ▷ Bottom nodes (leaves) of the tree contains class labels to be associated with the subsequences
 - ▷ Dynamic programming is used to deduce the complete gene structure

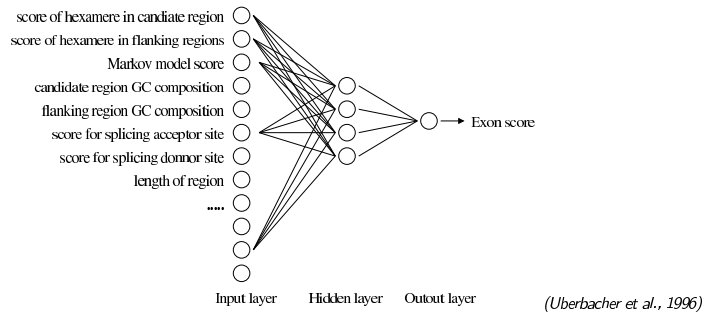


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Integrating signal information and compositional information for gene structure prediction (3)

A non exhaustive list of the available methods (continuation):

- Neural network
 - ▷ The neural network is trained with a set of true positives and true negatives examples
 - ▷ For each training example, the neurons are tuned to return the right answer
 - ▷ Dynamic programming is used to deduce the complete gene structure



Integrating signal information and compositional information for gene structure prediction (4)

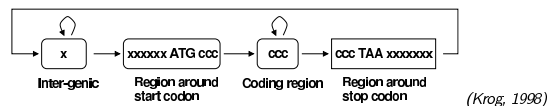
A non exhaustive list of the available methods (continuation):

- Markov Model (MM)
 - ▷ Biological sequences can be modeled as the output of a stochastic process in which the probability for a given nucleotide to occur at position p depends on the k previous positions. This representation is called k -order Markov Model.

$$P(x_i|x_1, x_2, \dots, x_{i-1}) = P(x_i|x_{i-k}, x_{i-(k-1)}, \dots, x_{i-1})$$



- Hidden Markov Model (HMM)
 - ▷ In a HMM the biological sequences are modeled as the output of a stochastic process that progresses through a series of discrete states. Each state model correspond to a Markov Model.



Integrating signal information and compositional information for gene structure prediction (5)

A non exhaustive list of the available methods (continuation):

- Generalized Hidden Markov Model (GHMM)
 - ▷ GHMMs are HMMs where states are arbitrary sub-models (e.g., neural networks, position weight matrices, etc.).
 - ▷ The duration of a particular state depends on some probability distributions.

Principle of Markov Models

- Given a DNA string S , find the most probable path M in the model that generates S . This will be the most probable gene structure.

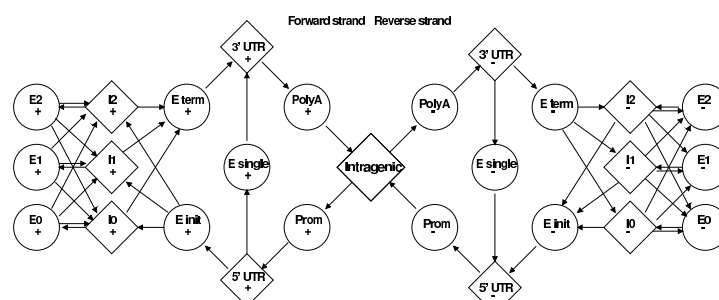
Markov derived models have many desirable properties

- Modeling: theoretically well-founded models.
- Efficient: $O(|M| \cdot |S|)$ where $|M|$ is the number of states in the model and $|S|$ is the length of the string.
- Scoring: theoretically well-founded scoring system.

Integrating signal information and compositional information for gene structure prediction (5)

Example of the GHMM in GENSCAN (*Burge and Karlin, 1997*)

- Each state can correspond to a position weight matrix, a neural network, ...
- Each state has an associated length distribution (time) → there are no transitions from the state to the state itself.
- The underlying (hidden) model of GENSCAN:



GRAIL

GRAIL 1

- Neural network recognizing coding potential within a fixed-size window (100 bases)
- Evaluates coding potential without looking for additional features (*e.g.*, splice junctions, start/stop codons)
- Exon quality evaluated by a score

GRAIL 1a

- Look at regions immediately adjacent to regions with coding potential
- Determine the "best" boundaries for the coding region
- Performs better than GRAIL 1 in finding true exons and eliminating false positives
- Returns a score

GRAIL (2)

GRAIL 2

- Uses variable window length
- Incorporates genomic context information
 - ▷ Splice junctions
 - ▷ Start and stop codons
 - ▷ PolyA signals
- Requires regions next to an exon
- Not appropriate for sequences without genomic context
- Much better at estimating the true extent of an exon as compared to GRAIL 1
- Returns scores
- WEB server
 - ▷ <http://compbio.ornl.gov>
 - ▷ Accept multiple sequence
 - ▷ Length 100 bases to 100 kilobases
 - ▷ Available for Human, Mouse, Drosophila, Arabidopsis, and E. coli

GRAIL (3)

GRAIL 2 output

- [grail2exons -> Exons]

	St	Fr	Start	End	ORFstart	ORFend	Score	Quality
1-	f	1	479	666	452	670	52.000	good
2-	f	0	5176	5290	5176	5370	82.000	excellent
3-	f	2	5396	5562	5364	5618	99.000	excellent
4-	f	0	7063	7113	7063	7113	53.000	good
5-	f	0	11827	11899	11590	11925	74.000	good
6-	f	0	12188	12424	12163	12633	88.000	excellent
7-	f	0	14288	14623	14194	14640	94.000	excellent
8-	f	0	17003	17203	16967	17235	100.000	excellent
9-	f	0	17751	17859	17659	17988	50.000	good
10-	f	1	18212	18264	18071	18268	61.000	good

- [grail2exons -> Exon Translations]

11- MLRGIDASNSEVFKKAKIMFLEVRKSLTCCQPTGSSCNAGQRESGHA
AFGIKHTQSVDR

12- AQIPNQQLKETTMCRAISLRLLLLLQLCKFSDLGT

13- AQLLAVTQGKTLVLGKEGESAEPLCESSQKKITVFTWKFSDQRKILGQH
KGVLIR

...

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FGENES

Linear discriminant analysis

- Combine several measures of pattern recognition using a linear discriminant analysis
 - ▷ Donor and acceptor splice sites
 - ▷ Putative coding regions
 - ▷ 5' and 3' intronic regions of the putative exon
- Pass the previous results to a dynamic programming algorithm to find a coherent gene model

Each predicted exon has an associated score, but not directly useful (most of the scores < 10, while probable good prediction scores > 10)

WEB server

- <http://genomic.sanger.ac.uk/gf/gf.shtml>
- Can combine homology method with ab initio results
- Available organisms
 - ▷ Human, Drosophila, Worm, Yeast, Plants

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FGENES (2)

FGENES output (CDSf = first exon; CDSi = internal exon; CDSl = last exon; CDSo = only one exon; PoA = PolyA signal)

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● Length of sequence: 20000 GC content: 0.48 Zone: 2
Number of predicted genes: 2 In +chain: 2 In -chain: 0
Number of predicted exons: 12 In +chain: 12 In -chain: 0
Predicted genes and exons in var: 2 Max var= 15 GENE WEIGHT: 27.3
G Str Feature Start End Weight ORF-start ORF-end

1 + 1 CDSf 990 - 1032 1.84 990 - 1031
1 + 2 CDSl 1576 - 1835 0.89 1578 - 1832
1 + PoA 3106 4.64

2 + 1 CDSf 5215 - 5266 5.25 5215 - 5265
2 + 2 CDSi 5395 - 5562 3.08 5397 - 5561
2 + 3 CDSi 11464 - 11490 0.76 11466 - 11489
2 + 4 CDSi 11738 - 11899 3.28 11740 - 11898
2 + 5 CDSi 12188 - 12424 2.48 12190 - 12423
2 + 6 CDSi 14288 - 14623 3.26 14290 - 14622
2 + 7 CDSi 17003 - 17203 2.79 17005 - 17202
2 + 8 CDSi 17741 - 17859 1.62 17741 - 17857
2 + 9 CDSi 18197 - 18264 2.53 18196 - 18264
2 + 10 CDSl 18324 - 18630 0.87 18325 - 18627

Predicted proteins:
>FGENES-M 1.5 >MySeq 1 Multiexon gene 990 - 1835 100 a Ch+
MSSAFSDPFKEQNPVISLITRIMLNSSSLPVRIYQPPMFLYIAPCAVLVLTSTSTPRR
TENGPLRMALNSRFPASFYLLCRDYQYTPPQLGPLHGRCS
>FGENES-M 1.5 >MySeq 2 Multiexon gene 5215 - 18630 558 a Ch+
MCRAISLRRLLLLLLQLSLLAVTQKTLVLGKEGESAELPCESSQKTIIVFTWKFSDQR
...

```

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MZEF

- Designed to predict only internal coding exons
- To discriminate between coding and non-coding regions, MZEF uses "quadratic discriminant analysis" of different measures
 - ▷ Exon length
 - ▷ Intron-exon transition/Exon-intron transition
 - ▷ Branch-site scores
 - ▷ 5' and 3' splice sites scores
 - ▷ Exon score
 - ▷ Strand score
- Possible to set a prior probability depending on the gene density and G+C content
- Scores over-estimate of the accuracy of the prediction
- WEB server
 - ▷ <http://www.cshl.org/genefinder>
 - ▷ Length up to 200 kilobases
 - ▷ Available organisms: Human, Mouse, Arabidopsis, Fission yeast

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MZEF (2)

MZEF output

- Internal coding exons predicted by MZEF
Sequence_length: 19920 G+C_content: 0.475

Coordinates	P	Fr1	Fr2	Fr3	Orf	3ss	Cds	5ss
5315 - 5482	0.580	0.623	0.528	0.585	122	0.506	0.608	0.552
6475 - 6582	0.752	0.482	0.563	0.558	221	0.505	0.567	0.598
11658 - 11819	0.822	0.476	0.569	0.497	211	0.554	0.560	0.651
14208 - 14543	0.903	0.593	0.619	0.469	212	0.497	0.603	0.575

- Description of the symbols
 - ▷ P: Posterior probability (between .5 to 1.)
 - ▷ Fr_i: Frame preference score for the *i*th frame of the genomic sequence
 - ▷ Orf: ORF indicator, "011" (or "211") means 2nd and 3rd frames are open
 - ▷ 3ss: Acceptor score
 - ▷ Cds: Coding preference score
 - ▷ 5ss: Donor score

HMMgene

Designed to predict complete gene structure

Uses HMM with a criterion called "Conditional Maximum Likelihood" which maximize the probability of correct predictions

Can return sub-optimal prediction to help identifying alternative splicing

Regions of the sequence can be locked as coding and non-coding by the user

Probability score for each predicted exon

WEB server

- <http://genome.cbs.dtu.dk/services/HMMgene>
- Available organisms:
 - ▷ Human and worm

HMMgene (2)

HMMgene output

```

• # SEQ: Sequence 20000 (-) A:5406 C:4748 G:4754 T:5092
Sequence HMMgene1.1a firstex 17618 17828 0.578 - 1 bestparse:cds_1
Sequence HMMgene1.1a exon_1 17049 17101 0.560 - 0 bestparse:cds_1
Sequence HMMgene1.1a exon_2 14517 14607 0.659 - 1 bestparse:cds_1
Sequence HMMgene1.1a exon_3 13918 13973 0.718 - 0 bestparse:cds_1
Sequence HMMgene1.1a exon_4 12441 12508 0.751 - 2 bestparse:cds_1
Sequence HMMgene1.1a lastex 7045 7222 0.893 - 0 bestparse:cds_1
Sequence HMMgene1.1a CDS 7045 17828 0.180 - . bestparse:cds_1
Sequence HMMgene1.1a DON 19837 19838 0.001 - 1
Sequence HMMgene1.1a START 19732 19734 0.024 - .
Sequence HMMgene1.1a ACC 19712 19713 0.001 - 0
Sequence HMMgene1.1a DON 19688 19689 0.006 - 1
Sequence HMMgene1.1a DON 19686 19687 0.004 - 0
...

```

	position	prob	strand	and frame
firstex	17618	0.578	-	1
exon_1	17049	0.560	-	0
exon_2	14517	0.659	-	1
exon_3	13918	0.718	-	0
exon_4	12441	0.751	-	2
lastex	7045	0.893	-	0
CDS	7045	0.180	-	.
DON	19837	0.001	-	1
START	19732	0.024	-	.
ACC	19712	0.001	-	0
DON	19688	0.006	-	1
DON	19686	0.004	-	0

- Symbols: firstex = first exon; exon-*n* = internal exon; lastex = last exon; singleex = single exon gene; CDS = coding region

GENSCAN

Designed to predict complete gene structure

Uses generalized hidden Markov models (GHMM): structure of genomic sequence is modeled by explicit state duration HMM

Signals are modeled by weight matrices, weight arrays, and maximal dependence decomposition

Probability score for each predicted exon

WEB server

- <http://genes.mit.edu/GENSCAN.html>
- Sequence length up to 200 kilobases
- Graphical output available
- Available organisms

▷ Vertebrate, Arabidopsis, Maize

GENSCAN (2)

GENSCAN output

```

● Gn.Ex Type S .Begin ...End .Len Fr Ph I/Ac Do/T CodRg P.... Tscr..
-----
1.00 Prom + 1653 1692 40 -1.16
1.01 Init + 5215 5266 52 0 1 83 75 151 0.925 12.64
1.02 Intr + 5395 5562 168 2 0 89 75 163 0.895 15.02
1.03 Intr + 11738 11899 162 0 0 74 113 101 0.990 11.15
1.04 Intr + 12188 12424 237 0 0 71 86 197 0.662 15.39
1.05 Intr + 14288 14623 336 0 0 82 98 263 0.986 22.19
1.06 Intr + 17003 17203 201 0 0 116 86 102 0.976 12.06
1.07 Intr + 17741 17859 119 0 2 78 109 51 0.984 6.38
1.08 Intr + 18197 18264 68 1 2 103 72 81 0.541 5.70

```

```

>02:36:44|GENSCAN_predicted_peptide_1|448_aa
MCRAISLRRLLLLLLQLSQLLAVTQKTLVLGKGESEAELPCESSQKKITVFTWKFSDQR
KILGQHGGVLRGGSPSDFDRFDSKKGAWERKGSFPLIINKLKMEDSQITYICELENRKEE
...

```

```

Gn.Ex : gene number, exon number (for reference)
Type : Init = Initial exon (ATG to 5' splice site)
      Intr = Internal exon (3' splice site to 5' splice site)
      Term = Terminal exon (3' splice site to stop codon)
      Sngl = Single-exon gene (ATG to stop)
      Prom = Promoter (TATA box / initiation site)
      PlyA = poly-A signal (consensus: AATAAA)
S      : DNA strand (+ = input strand; - = opposite strand)
Begin  : beginning of exon or signal (numbered on input strand)
End    : end point of exon or signal (numbered on input strand)
Len    : length of exon or signal (bp)
Fr     : reading frame (a forward strand codon ending at x has frame x mod 3)

```

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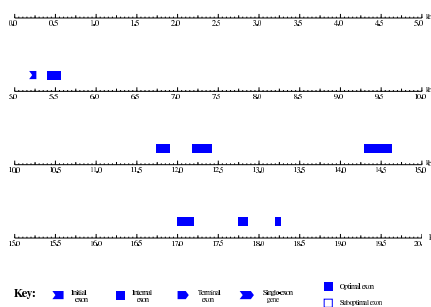
```

Ph      : net phase of exon (exon length modulo 3)
I/Ac   : initiation signal or 3' splice site score (tenth bit units)
Do/T   : 5' splice site or termination signal score (tenth bit units)
CodRg  : coding region score (tenth bit units)
P      : probability of exon (sum over all parses containing exon)
Tscr   : exon score (depends on length, I/Ac, Do/T and CodRg scores)

```

● Graphical output

GENSCAN predicted genes in sequence 02:36:44



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GeneMark.hmm

Initially developed for bacterial gene finding

Recently modified to predict gene structure in eukaryotes

Uses explicit state duration HMM

Optimal gene candidates, selected by HMM and dynamic programming, are further processed by a ribosomal binding site recognition algorithm

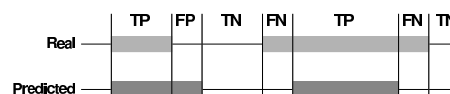
No scores!

WEB server

- <http://opal.biology.gatech.edu/GeneMark>
- Access to both the original GeneMark and GeneMark.hmm

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Evaluating performances



Measures (*Burset and Guigo, 1996; Snyder and Stormo, 1997*)

- **Sensitivity** S_n is the proportion of coding nucleotides that are correctly predicted as coding:

$$S_n = \frac{TP}{TP+FN}$$

- **Specificity** S_p is the proportion of nucleotides predicted as coding that are actually coding:

$$S_p = \frac{TP}{TP+FP}$$

- **Correlation coefficient** CC is a single measure that captures both specificity and sensitivity:

$$CC = \frac{(TP \times TN) - (FN \times FP)}{\sqrt{(TP+FN) \times (TN+FP) \times (TP+FP) \times (TN+FN)}}$$

- **Approximate correlation** AC is similar to CC , but defined under any circumstances:

$$AC = (ACP - 0.5) \times 2$$

where

$$ACP = \frac{1}{4} \left(\frac{TP}{TP+FN} + \frac{TP}{TP+FP} + \frac{TN}{TN+FP} + \frac{TN}{TN+FN} \right)$$

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Accuracy of the different methods

Evaluation of the different programs (*Rogic et al., 2001*)

Programs	No. of sequences	S_n	S_p	AC	CC
FGENES	195	0.86	0.88	0.84 ± 0.19	0.83
GeneMark.hmm	195	0.87	0.89	0.84 ± 0.18	0.83
GENSCAN	195	0.95	0.90	0.91 ± 0.12	0.91
HMMgene	195	0.93	0.93	0.91 ± 0.13	0.91
MZEF	119	0.70	0.73	0.68 ± 0.21	0.66

- Overall performances are the best for HMMgene and GENSCAN.
- Some program's accuracy depends on the G+C content, except for HMMgene and GENSCAN, which use different parameters sets for different G+C contents.
- For almost all the tested programs, "medium" exons (70-200 nucleotides long), are most accurately predicted. Accuracy decrease for shorter and longer exons, except for HMMgene.
- Internal exons are much more likely to be correctly predicted (weakness of the start/stop codon detection).
- Initial and terminal exons are most likely to be missed completely.
- Only HMMgene and GENSCAN have reliable scores for exon prediction.

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Gene prediction limits

Existing predictors are for protein coding regions

- Non-coding areas are not detected (5' and 3' UTR)
- Non-coding RNA genes are missed

Predictions are for "typical" genes

- Partial genes are often missed
- Training sets may be biased
- Atypical genes use other grammars

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