Searching for RNA genes using base-composition statistics

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ABSTRACT

The hypothesis that genomic regions rich in non-protein-coding RNAs (ncRNAs) can be identified using local variations in single-base and dinucleotide statistics has been investigated. (G+C)%, (G–C)% difference, (A–T)% difference and dinucleotide-frequency statistics were compared among seven classes of ncRNAs and three genomes. Significant variations were observed in (G+C)% and, in *Methanococcus jannaschii*, in the frequency of the dinucleotide 'CG'. Screening programs based on these two base-composition statistics were developed. With (G+C)% screening alone, a 1% fraction of the *M.jannaschii* genome containing all 44 known transfer RNAs, ribosomal RNAs and signal recognition particle RNAs could be identified. When (G+C)% combined with CG dinucleotide-frequency screening was used, 43 of the 44 known *M.jannaschii* structural ncRNAs were again identified, while the number of presumably false hits overlapping a known or putative protein-coding gene was reduced from 15 to 6. In addition, 19 candidate ncRNAs were identified including one with significant homology to several known archaeal RNaseP RNAs.

INTRODUCTION

Non-protein-coding RNAs (ncRNAs) are known to play significant roles in biological systems. Along with the familiar transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), ncRNAs contribute to gene splicing, RNA nucleotide modification, protein transport and regulation of gene expression (1). Consequently, identifying ncRNAs is an important task. Conventional protein gene-finding programs such as Genscan are not designed to locate ncRNAs. In cases where detailed characterization of the ncRNA gene families is possible, specialized RNA gene-finding programs have been quite successful (2–5). In addition, promising new ncRNA search algorithms based on comparative genomics have been proposed (6–8). Nevertheless, the goal of locating ncRNAs, when detailed characterizations of the target RNA sequences are unavailable, remains difficult to achieve.

One intriguing approach (9) is that, for some genomes, the local percentage of GC bases (G+C)% may serve as a filter to screen for ncRNA-rich regions. Genomic variations in base-composition statistics, such as (G+C)%, and their application to searching for protein-coding genes have been studied for many years (reviewed in 10). In particular, it is well known that thermophiles maintain the stability of ncRNAs by increasing their (G+C)% (11,12). It is also known that thermophiles generally use mechanisms other than (G+C)% elevation to maintain the stability of their genomic DNA (13,14). Based on these ideas, Rivas and Eddy proposed ncRNA gene-finding based on (G+C)% (9). They also suggested this approach may be applicable even in non-thermophiles—such as *Caenorhabditis elegans*—which have differing ncRNA and genomic (G+C)% (9). However, the feasibility of such an approach has not been clear since other investigations (15–17) have indicated that base composition alone is not sufficient to predict RNA folding and hence the occurrence of ncRNAs in the genome.

Other base-composition statistics besides (G+C)% might also be expected to vary between ncRNAs and their genomic background. For example, the single-strand G minus C excess, (G–C)%—also known as the 'G–C% Chargaff difference'—has long been known to be very small for a wide range of organisms (18). Similarly small values have been observed for (A–T)% Chargaff differences. Moreover, at least in *Escherichia coli*, these approximate G=C and A=T single-strand frequency equalities—known as 'Chargaff's Second Law'—have been observed on the local level as well (18).

On the other hand, RNAs may be subject to constraints causing deviation from Chargaff's Second Law. For example, recent work suggests that non-zero Chargaff differences may mark the presence of protein-coding messenger RNAs (mRNAs) (19). Moreover, G/U base pairs are more commonly found in RNA structure than C/A base pairs, which might lead to local violations of Chargaff's Second Law.

Relative dinucleotide frequencies might also be anticipated to vary between ncRNAs and the genomic background. Though dinucleotide frequencies vary widely among species, they tend to be relatively constant within the genome of any single species (10). However, ncRNAs are subject to structural constraints that may influence their dinucleotide composition. ncRNAs typically form folded structures whose stacking energies—and consequently whose conformation—depend on their dinucleotide composition (20–21). As a result, one might expect that ncRNA dinucleotide frequencies would display

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characteristic atypical values relative to the genomic background.

The present work investigates the feasibility of using local base-composition statistics to distinguish between ncRNA-rich and ncRNA-poor regions of the genome. The goal is to partition a genomic region into two components—one well defined component with high probability of containing ncRNAs and the other component with low probability of containing ncRNAs. To assess the feasibility of this approach, base-composition statistics from a variety of ncRNAs were compiled. Similar statistics were acquired from three test genomes (*Methanococcus jannaschii*, *Plasmodium falciparum* and *C.elegans*) and were compared with the values found from the RNA sequences. Lastly, for the case where the largest RNA-genome variations were observed—specifically, for (G+C)% and ρ(CG) in *M.jannaschii*—two programs were developed to apply these statistical variations to genome-wide searching for ncRNA genes. Using these programs led to the identification of 36 known tRNAs, six known rRNAs and one known signal recognition particle RNA in *M.jannaschii*. In addition, 19 putative ncRNAs including one with homology to several RNaseP RNAs were identified.

MATERIALS AND METHODS

Data sources

Sequence data for cytoplasmic tRNAs (22), rRNAs (23,24), riboregulator RNAs (25), small nuclear RNAs (snRNAs) and small cytoplasmic RNAs (http://mbcr.bcm.tmc.edu/smallRNA/), small nucleolar RNAs (snoRNAs) (http://rna.wustl.edu/snoRNAdb/), signal recognition particle RNAs (SRP RNAs) (26), and RNA pseudoknots (27) were obtained from the public databases.

Genomic data for *M.jannaschii*, *P.falciparum* and *C.elegans* were downloaded from the NCBI GenBank genomes databases (ftp://ftp.ncbi.nih.gov/genomes/) in April 2001.

Base-composition statistics calculated

For each sequence, the following statistics were computed:
(G+C)% = 100 $(n_G + n_C)/(n_A + n_C + n_G + n_T)$
(G–C)% Chargaff difference = 100 $(n_G - n_C)/(n_G + n_C)$
(A–T)% Chargaff difference = 100 $(n_A - n_T)/(n_A + n_T)$
$\rho(AB) = [f(AB)/f(A)*f(B)] = (L * n_{AB})/(n_A * n_B)$
where L is the sequence length, n_B and n_{AB} are the number of occurrences of base 'B' or the dinucleotide AB, respectively, and f(B) or f(AB) is the frequency of occurrence of base B or dinucleotide AB [e.g. $f(B) = n_B / L$]. [We use Karlin *et al.*'s (10) variable ρ rather than their variable ρ* to measure dinucleotide frequencies since we are interested in observing the effects of RNA genes that will be present on only one of the two DNA strands.]

It should be noted that a systematic, positive (G–C)% difference in ncRNAs would not necessarily imply a positive genomic (G–C)% difference at an ncRNA gene since the ncRNA gene might be on the negative strand. However, any systematic non-zero ncRNA Chargaff differences of either sign would imply non-zero genomic Chargaff differences near an ncRNA gene. Moreover, if the absolute value of the local Chargaff difference indicated the presence of an ncRNA, then the sign of the difference could serve as an indicator of the strand on which the ncRNA was located. Similarly, systematic ncRNA dinucleotide variations (except for dinucleotides that are their own reverse complements) would result in somewhat different genomic signatures depending on which strand the ncRNA were located.

Base-composition statistical averages

RNA data were grouped by RNA type and by species. In most cases, *M.jannaschii*, *P.falciparum*, *C.elegans* and *Homo sapiens* RNA sequences were used. For those RNA classes for which the databases contained limited or no data for *M.jannaschii*, *P.falciparum*, *C.elegans* and *H.sapiens*, RNA sequence data from other organisms or groups of organisms were used, as noted in the text and tables.

For genomic averages, 1000 random samples of 100 bp each were selected per chromosome. In addition, tests were performed to check for any local base-composition variations along the length of a chromosome. For example, for chromosome I of *C.elegans*, the entire chromosomal sequence was divided into 49 regions of 294 kb each, and base-composition statistics for 1000 random samples of 100 bp were calculated for each of the 49 regions.

For each group of sequences (whether RNAs or genomic samples), means and standard deviations (SDs) of each of the base-composition statistics were calculated. For the genomic sequences, parameter means and SDs for each of the chromosomal subregions were also calculated. Statistical significance of population differences between parameter-means was determined using Student *t*-test comparisons with 95% confidence levels.

Algorithms and training of ncRNA search programs

Two ncRNA search algorithms were implemented. The first approach ('Program I') is based solely on scoring local (G+C)% values. (G+C)% of a subsequence is scored using a log odds (LOD) score (9):
$\mathrm{LOD} = C_{AT}(n_A + n_T) + C_{GC}(n_C + n_G)$
where
$C_{AT} = \log_2$[(A+T)% in RNA genes/(A+T)% in genome]
and
$C_{GC} = \log_2$[(G+C)% in RNA genes/(G+C)% in genome]

The second search algorithm ('Program II')identifies possible ncRNAs using a combination of (G+C)% and ρ(CG) values. The (G+C)% component of this algorithm is identical to Program I; however, any putative hit must also have a value of ρ(CG) larger than a specified cut-off value.

Both programs have a few adjustable parameters: the window lengths to be tried while searching for a hit, the minimum RNA length, and the LOD cut-off scores to indicate the beginning and end of a 'hit'. Program II has one additional parameter: the cut-off value for ρ(CG).

The adjustable parameters were chosen by training the programs on a 20 kb subsequence of the *M.jannaschii* sequence (from 850 000 to 870 000) which has six annotated tRNA genes and one annotated rRNA gene. Using this training data, the following parameter values were selected for Program I (and subsequently used for the scan of the entire genome): minimum RNA length = 40 bp; window length range = 25–100 bp; minimum LOD value for the start of a hit = 19; and minimum LOD value for the continuation of a hit = 13. After training Program II with the same training set, the cut-off value for

Table 1. Base-composition statistics for RNAs and genomes

	No. of sequences used	(G+C)%	ρ(CG)	(G–C)% difference	(A–T)% difference
(A) Average RNA base-composition statistics					
M.jannaschii	48	63.1 (7.3)	0.75 (0.24)	8.1 (9.7)	–3.3 (12.9)
Plasmodium	59	32.1 (7.2)	0.94 (0.56)	12.7 (6.3)	–1.6 (4.1)
C.elegans	59	53.5 (8.2)	0.96 (0.23)	6.8 (10.1)	–9.6 (11.4)
H.sapiens	186	48.7 (9.1)	0.60 (0.41)	7.5 (11.8)	–5.8 (13.0)
(B) Genomic base-composition statistics					
M.jannaschii		31.4 (6.9)	0.34 (0.47)	1.4 (36.9)	–0.34 (18.8)
P.falciparum Chr. II		20.0 (8.4)	0.75 (1.3)	0.73 (34.5)	–1.7 (24.0)
C.elegans Chr. I		35.9 (8.8)	1.03 (0.68)	0.65 (25.0)	–0.61 (19.6)

This table summarizes the differences in mean-value base-composition statistics between ncRNAs and the genomic background in *M.jannaschii*, *P.falciparum* and *C.elegans.* SDs are shown in parentheses. Statistics for RNAs of several *Plasmodium* species were averaged together since there are only a limited number of *P.falciparum* RNA sequences in the RNA databases. (A) Average base-composition statistics among ncRNAs. The low (32.1%) value for (G+C)% in *Plasmodium* in contrast to the high (>48%) (G+C)% value for the other genomes is striking. One also notes the positive (G–C)% values and negative (A–T)% values, possibly resulting from the occurrence of G-U 'wobble' pairs in the ncRNAs. (B) Base-composition statistics for three test genomes: *M.jannaschii*, *P.falciparum* chromosome II and *C.elegans* chromosomes (results for other *C.elegans* and *P.falciparum* chromosomes were similar—data not shown). One notes the differences in genome mean values from the RNA values of (G+C)% for *M.jannaschii* and *C.elegans* and for ρ(CG) for *M.jannaschii*. Data for dinucleotide frequencies other than ρ(CG) did not show systematic differences between RNAs and the genomic background (data not shown). Genomic (G–C)% and (A–T)% differences are seen to be very close to zero (as expected from 'Chargaff's Second Law') which is different from the RNA values shown in (A). However, the table also shows the large SDs for (G–C)% and (A–T)% relative to their mean values, implying that using these differences to distinguish RNAs from the background would be difficult.

ρ(CG) was set equal to 0.5 and the minimum LOD value for the start of a hit was set equal to 15, with the other parameters having the same values they had for Program I.

Assessment of the search programs

Both search programs were run against the *M.jannaschii* genome with the resulting hits compared with known RNAs as annotated in the GenBank '.gbk' database file at ftp://ftp.ncbi.nih.gov/genomes/. Sequence segments that scored above the scoring threshold and that did not overlap any GenBank annotated gene—as well as 60 bp extensions of those sequences—were input to the NCBI BLAST program (28) and run against the NCBI non-redundant nucleotide database (NRDB). In most cases, default BLAST parameters were used. A few BLAST searches were performed with non-default wordsize, match or mismatch values. Changing the BLAST parameter values did not result in any additional, noteworthy BLAST hits.

RESULTS

Two significant base-composition variations were observed between RNAs and the background genome: (G+C)% and, in *M.jannaschii*, ρ(CG). For example, in *M.jannaschii* RNA mean (G+C)% is 63.1%, while background (G+C)% is 31.4% (Table 1). In *C.elegans* RNA mean (G+C)% is 53.5%, while background (G+C)% (on chromosome I) is 35.9%. ρ(CG) in *M.jannaschii* RNAs is 0.75 while background ρ(CG) is 0.34 (Table 1). These differences between RNA and genome values for (G+C)% and ρ(CG) in *M.jannaschii* formed the basis for the RNA gene-finding algorithms.

However, we observed that RNA (G+C)% is not elevated in all species: *Plasmodium*-RNA mean (G+C)% is 32.1% (Table 1) indicating that—despite its low background (G+C)% (e.g. 20.0% for *P.falciparum* chromosome II)—*Plasmodium* is not a promising candidate for RNA gene-finding based on (G+C)%.

We also observed consistently positive ncRNA (G–C)% values and negative (A–T)% values (Table 1). It is possible that these result from the occurrence of G-U 'wobble' pairs in the ncRNAs. However, the differences between RNA and the background values for the Chargaff differences as well as for the dinucleotide variations—other than for ρ(CG)—were all smaller than, or comparable with, the corresponding individual population SDs (Tables 1 and 2; not all data shown). Consequently, these parameters were deemed unsuitable for a ncRNA gene-finder.

The results of testing Programs I and II against the *M.jannaschii* genome are shown in Tables 3 and 4. Both programs identify a set of sequence regions that are predicted to contain a high percentage of structural RNAs. Together, these regions consist of <1% of the *M.jannaschii* genome. With Program I, this 1% subsequence contains all 43 annotated tRNAs and rRNAs in the GenBank '.gbk' file as well as the single *M.jannaschii* SRP RNA in the SRP RNA database (20). Program II has slightly lower sensitivity, finding 43 of the 44 annotated tRNAs, rRNAs and SRP RNAs [a tRNA at location 637 982 with ρ(CG) = 0.28 is missed].

Table 2. Variations in (C+G)% and ρ(CG) statistics among differing RNA classes

RNA	Species	(C+G)%	ρ(CG)
tRNAs (cytoplasmic)	*M.jannaschii*	66.2	0.72
	Plasmodium	25.6	0.88
	C.elegans	58.8	0.92
	H.sapiens	58.0	1.00
rRNAs	*M.jannaschii*	63.8	0.87
	Plasmodium	36.0	0.98
	C.elegans	48.0	1.04
	H.sapiens	60.3	1.03
SRP RNAs	*M.jannaschii*	65.7	0.66
	C.elegans	56.8	1.07
	H.sapiens	58.3	0.48
Small nuclear RNAs	*C.elegans*	43.1	1.01
	H.sapiens	44.3	0.90
sno- and sno-like RNAs	*M.jannaschii*	48.7	0.83
	H.sapiens	44.2	0.38
Riboregulator RNAs	*H.sapiens*	47.6	0.33
	All eukaryotes	44.4	0.46
Pseudoknot RNAs	All bacteria	52.0	0.86
	All viruses	51.8	0.98

This table shows some of the variations in (C+G)% and ρ(CG) values among different classes of RNAs for *M.jannaschii*, *Plasmodium* (multiple species combined because of limited data), *C.elegans* and *H.sapiens*. For the riboregulator and pseudoknot databases, which contain limited or no data for these species, RNA sequence data from other groups of organisms are shown. The base-composition variations shown in the table indicate which ncRNA types are more likely to be detected by a RNA gene-finder based on (C+G)% and ρ(CG). For example, one notes that (C+G)% is only 48.7% in *M.jannaschii* snoRNAs and 43.1% in *C.elegans* snRNAs. Consequently these RNAs would be more difficult to detect using a (C+G)% detector than the more G+C rich tRNAs, rRNAs and SRP RNAs.

The primary difference between the results of the two programs is that Program I yields nine more ‘hits’ that overlap a putative protein-coding gene than does Program II (Table 3). Running NCBI BLASTX searches against the NCBI non-redundant protein database with these nine sequences showed that five of them have strong homologies (E < 10e–5) to exons of multiple (five or more) other species (data not shown). This result suggests that most—if not all—of the nine additional overlapping hits are false-positives for ncRNAs, which in turn implies that using ρ(CG) together with (G+C)% results in higher specificity than using (G+C)% alone. In contrast, when the six sequences identified by both Program I and Program II that overlap putative protein genes were run with BLASTX, only one showed significant homology to exons of other species.

In addition to finding the known tRNAs, rRNAs and SRP RNAs, Program II identified 22 additional ‘hits’. Among these hits were three pairs of hits located within 40 bp of each other and likely to be parts of three longer ncRNAs (shown as cnr1, cnr7 and cnr12 in Table 4). Consequently there are a total of 19 candidate, novel ncRNAs which are listed with their genomic start and end locations in Table 4. Since these locations were determined solely on the basis of elevated (C+G)% and ρ(CG), they indicate only the approximate end points of the candidate ncRNAs. A predicted strand is not determined by the present methods, since (C+G)% and ρ(CG) are identical for both strands. BLAST searches with these 19 sequences identified one sequence, cnr10 in Table 4, as homologous (E-values < 10e–11) to RNaseP RNAs (rnpB) of *Methanococcus vannielii*, *Methanococcus thermolithotrophicus* and *Methanococcus maripaludis*. Figure 1 shows a Clustal W (29) multiple alignment of cnr10 and its surrounding sequence with the three homologous RNaseP genes.

A next step in determining which, if any, of the candidate ncRNAs is real might be to look for promoter and terminator sequences adjacent to the putative RNAs in a manner similar to the method of Argaman *et al.* (8). However, archaeal promoters and terminators are far less well characterized than those in bacteria, and consequently we have not attempted to implement such a search. Experimental testing—such as northern analyses or microarray expression profiling under various growth conditions—is needed to determine whether these putative ncRNAs are transcribed.

DISCUSSION

The present work has shown that in *M.jannaschii* it is possible to locate structural ncRNAs solely on the basis of local variations of genomic base composition. In *M.jannaschii*, these base-composition variations led to identifying 36 known tRNAs, six known rRNAs and one known SRP RNA. In addition, 19 putative ncRNAs including one with homology to several RNaseP RNAs were identified. Although the method worked well at identifying tRNAs, rRNAs and SRP RNAs—

Table 3. Results of (G+C)% + ρ(CG) screening on *M.jannaschii*

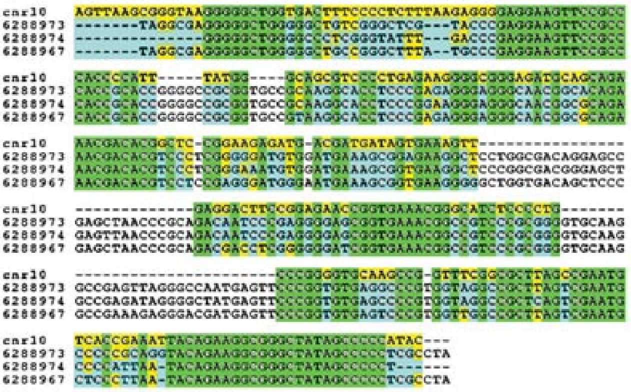
Method	Annotated RNAs found	Annotated RNAs missed	Total no. of other hits	Other hits overlapping >50% putative protein gene	Putative protein genes with BLASTX hits to multiple species
(G+C)% alone	44	0	41	15	6
(G+C)% and ρ(CG)	43	1	28	6	1

Summary of results of scanning the *M.jannaschii* genome for ncRNAs using (G+C)% or (G+C)% combined with ρ(CG). Annotated RNAs include tRNAs, rRNAs and SRP RNAs of which only six tRNA genes and one rRNA gene were included in the training set. One notes adding ρ(CG) screening causes one RNA to be missed while reducing the number of putative hits overlapping a protein gene from 15 to 6, of which only one shows homology using BLASTX to exons from multiple other species. Further details are in the text.

Table 4. Candidate *M.jannaschii* ncRNAs

ID no.	Genomic start position	Genomic end position	G+C%	ρ(CG)
cnr1	118060	118190	54.8	0.95
cnr2	291952	291992	63.4	1.16
cnr3	325022	325062	53.7	1.00
cnr4	412566	412606	56.1	0.63
cnr5	465392	465533	61.0	0.51
cnr6	471565	471605	61.0	0.56
cnr7	537593	537711	56.1	0.86
cnr8	543830	543896	53.0	0.86
cnr9	638309	638411	56.9	0.72
cnr10	643488	643698	57.6	0.71
cnr11	873579	873622	53.5	0.64
cnr12	951834	951959	56.8	0.68
cnr13	986076	986116	61.0	1.11
cnr14	1129093	1129170	52.0	0.90
cnr15	1131997	1132037	53.7	1.03
cnr16	1137151	1137191	56.1	0.63
cnr17	1204222	1204262	56.1	1.33
cnr18	1606179	1606219	63.4	0.75
cnr19	1659426	1659497	50.0	0.69

List of 19 candidate ncRNAs identified by combined (G+C)% + ρ(CG) screening. Positions along the *M.jannaschii* genome as well as values for the statistical parameters are shown. Strand location is not determined by the present methods, since (C+G)% and ρ(CG) are identical for both strands. Three of the candidate ncRNAs (cnr1, cnr7 and cnr 12) consist of two immediately-adjacent 'hits' presumably resulting from a single ncRNA and combined in the table. cnr10 is homologous to several RNaseP genes.



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cnr10   AGTTAAGCGGGTAAGGGGGCTGGTGACTTTCCCCTCTTTAAGAGGGGAGGAAGTTCCGCC
6288973 -------TAGGCGAGGGGGCTGGGGGCTGTCGGGCTCG---TACCCGAGGAAGTTCCGCC
6288974 --------------GGGGGCTGGGGCCCTCGGGTATTT---GACCCGAGGAAGTTCCGCC
6288967 -------TAGGCGAGGGGGCTGGGGGCTGCCGGGCTTTA--TGCCCGAGGAAGTTCCGCC

cnr10   CACCCCATT-----TATGG----GCAGCGTCCCCTGAGAAGGGGCGGGAGATGCAGCAGA
6288973 CACCGCACCGGGGCCGCGGTGCCGCAAGGCACCTCCCGAGAGGGAGGGCAACGGCACAGA
6288974 CACCGCACCGGGGCCGCGGTGCCGCAAGGCACCTCCCGGAAGGGAGGGCAACGGCGCAGA
6288967 CACCGCACCGGGGCCGCGGTGCCGTAAGGCACCTCCCGAGAGGGAGGGCAACGGCGCAGA

cnr10   AACGACACGGCTC-CGGAAGAGATG-ACGATGATAGTGAAAGTT----------------
6288973 AACGACACGTCCCTCGGGGGATGTGGATGAAAGCGGAGAAGGCTCCTGGCGACAGGAGCC
6288974 AACGACACGTCCCTCGGGAAATGTGGATGAAAGCGGTGAAGGCTCCCGGCGACGGGAGCT
6288967 AACGACACGTCCTCCGAGGGATGGGAATGAAAGCGGTGAAGGGGGCTGGTGACAGCTCCC

cnr10   -------------GAGGACTTCCGGAGAACCGGTGAAACGGGCATCTCCCCTG-------
6288973 GAGCTAACCCGCAGACAATCCCGAGGGGAGCGGTGAAACGGCCGTCCCGCGGGGTGCAAG
6288974 GAGTTAACCCGCAGACAATCCCGAGGGGAGCGGTGAAACGGCCGTCCCGCGGGGTGCAAG
6288967 GAGCTAACCCGCAGACGACCTCGGGGGGATCGGTGAAACGGCCGTCCCGCGGGGTGCAAG

cnr10   ----------------------CCCGGGGTGCAAGCCG-GTTTCGGCGCTTAGCCGAATG
6288973 GCCGAGTTAGGGCCAATGAGTTCCCGGTGTGAGGCCCGTGGTAGGCCGCTTAGTCGAATG
6288974 GCCGAGATAGGGGCTATGAGTTCCCGGTGTGAGCCCCGTGGTAGGCCGCTCAGTCGAATG
6288967 GCCGAAAGAGGGACGATGAGTTCCCGGTGTGAGTCCCGTGGTTGGCCGCTTAGTCGAATG

cnr10   TCACCGAAATTACAGAAGGCGGGCTATAGCCCCCATAC---
6288973 CCCCCGCAGGTACAGAAGGCGGGCTATAGCCCCCTCGCCTA
6288974 CCCCATTAA-TACAGAAGGCGGGCTATAGCCCCCT------
6288967 CTCCCTTAA-TACAGAAGGCGGGCTATAGCCCCCTCGCCTA
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Figure 1. Alignment of cnr10 and RNaseP genes from *M.vannielii* (AF192357/GBBCT:6288966), *M.thermolithotrophicus* (AF192355/GBBCT:6288964) and *M.maripaludis* (AF192354/GBBCT:6288963). The RNaseP sequences are partial sequences obtained from the specified GenBank records.

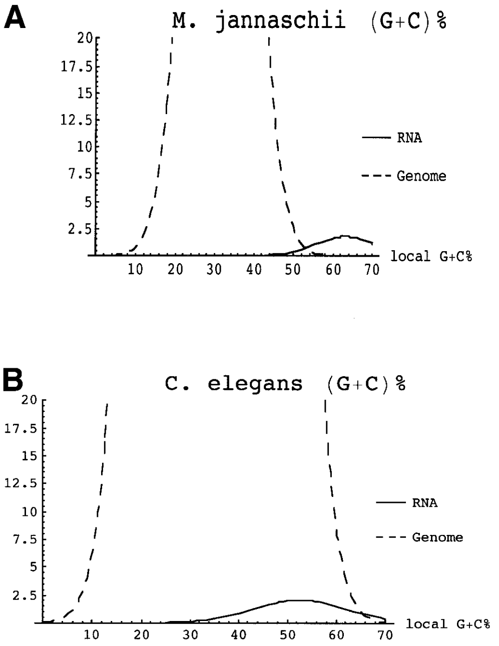

Figure 2. Separation of RNAs and genomic background using G+C%. Vertical axes indicate estimated relative number of 100 bp subsequences. Note that the peak of the curve for the number of genomic sequences is truncated. RNA estimate assumes ratio of protein coding genes to ncRNA genes is approximately equal to that in *S.cerevisiae*. Graphs are shown as normally distributed for the purpose of illustration—the actual distribution of G+C% may vary. (**A**) *M.jannaschii* RNA and genome G+C% distributions are separated enough to enable discrimination between RNA and background populations. (**B**) *C.elegans* chromosome X. Although *C.elegans* ncRNA and genomic G+C% population means are significantly different, ncRNA distribution cannot be distinguished from that of the background by G+C% alone.

which all have mean RNA (G+C)% >60%—it failed to find any of the eight *M.jannaschii* C-D snoRNA-like genes. These RNAs have mean (G+C)% = 49% (Table 2). Similarly, one notes in Table 2 that for snRNAs (G+C)% is 43.1% in *C.elegans* and 44.3% in *H.sapiens*. For riboregulator RNAs, (G+C)% is 47.6% in *H.sapiens* and 44.4% in all eukaryotes. Consequently, the present method is also likely to be less successful at identifying snRNAs and riboregulator RNAs.

It would be desirable if the present approach could be applied to additional genomes. However, this will be challenging, since most genomes have higher background (G+C)% than *M.jannaschii.* For example, background genomic (G+C)% is ~34% in *C.elegans* and 39% in *Saccharomyces cerevisiae*, compared with 29% in *M.jannaschii* (9). Although the mean genomic (G+C)% value of *C.elegans* (34%) is still quite different from the *C.elegans* RNA (G+C)% values (~53%, see Table 1), the problem is that the random fluctuations of genomic (G+C)% are also large (~8% SDs, see Table 1). Consequently, finding ncRNAs in *C.elegans* on the basis of (G+C)% alone is not feasible. Figure 2 illustrates the problem. The situation in *S.cerevisiae* is even less encouraging, since background (G+C)% = 39%, while typical (G+C)% for tRNAs is only 54% (9). Even in *P.falciparum*—which initially appeared promising because of its low mean background (G+C)% of 20%—identifying ncRNAs using (G+C)% is not likely to be feasible because of the low mean RNA (G+C)% of 32% in *Plasmodium* species (Table 1). Consequently, at this point, it appears that success with this method will be restricted to species like *M.jannaschii* which have high RNA (G+C)% (a constraint common among hyperthermophiles) as well as low genomic (G+C)%.

We had initially thought that other base-composition parameters—such as Chargaff differences and dinucleotide frequencies—might offer additional statistical signatures with which to differentiate ncRNAs from the background. However, with the exception of ρ(CG) in *M.jannaschii*, we were unable to find any other base-composition variations between ncRNAs and the background that were large enough to be usefully incorporated into an RNA gene-finder.

We had also hoped to improve our results by restricting the ncRNA searches to genomic regions with relatively high (A+T)%. This idea is based on evidence for 'isochores' in chromosomes of vertebrates (30), and, more recently, also in chromosomes of non-vertebrate eukaryotes (31). Isochores are 100–300 kb regions with relatively homogenous, local (G+C)% mean values which may vary by as much as 3–4% from the overall genomic (G+C)% mean (29). For example, we computed over a set of 49 regions of 100 kb on *C.elegans* chromosome I, that mean (G+C)% ranges from 33.3 to 40.3%. Consequently, (G+C)%-based ncRNA screening might be improved if the search was restricted to isochores with elevated (A+T)%. However, we have also found that isochore base-composition homogeneity does not extend down to the length of typical RNAs—i.e. ~100 bp. As a result, the strategy of focusing on high (A+T)% isochores appears unlikely to significantly improve the performance of (G+C)%-based screening programs.

The present approach can be compared with other recent methods for ncRNA gene-finding. The most similar is that of Klein and Eddy (http://ismb00.sdsc.edu/posters/posterlist.html) who use a hidden Markov model (HMM) based on (G+C)% alone to search for ncRNAs in *M.jannaschii* and other high (A+T)% thermophillic organisms. After the present work was completed and submitted for publication, Klein, Misulovin and Eddy presented a detailed description of their method and the results of an experimental search for the ncRNAs that they predict (ftp://ftp.genetics.wustl.edu/pub/eddy/papers/2002-klein-archaea/preprint.pdf).

There are three principal differences between their approach and the one taken in this work. First, Klein's model is based solely on (G+C)% variations. By additionally using ρ(CG), the present method eliminates potential false positives that overlap known and putative protein-coding regions. Secondly, different assumptions were made regarding ncRNA lengths. In the present work, the only ncRNA length restriction is that 'hits' must be $\geq$40 bases long. Klein *et al.* make more restrictive assumptions—not only must the ncRNA length be $\geq$50 bases, but they also set their HMM transition probabilities so that the average ncRNA length will be approximately 100 bases. Finally, Klein's approach is implemented using an HMM while the present method uses a scanning, base-counting window. Although fundamentally similar, algorithm operation and dependence on model parameters are easier to interpret in base-counting methods than in HMMs. Specifically, in addition to the ncRNA length and base-count cut-offs common to both approaches, HMMs require a set of transition-probability parameters; determining the sensitivity of the HMM results to varied assumptions in these parameters may not be easy to predict.

It is interesting to compare Klein *et al.*'s predictions and experimental results with the predictions of the present work. Klein *et al.* experimentally verify four of the nine *M.jannaschii* ncRNAs that they predict. We note that all four of the experimentally confirmed ncRNAs are also identified by the present approach (Klein's mja2, mja3, mja6 and mja7 correspond to our cnr1, cnr3, cnr12 and cnr14, respectively). In addition, two other unconfirmed candidates (Klein's mja4 and mja9, corresponding to our cnr4 and cnr19) are predicted by both approaches. Klein's mja1 is not predicted by the present work because it is located on the *M.jannaschii* extrachromosomal segment which was not analyzed in the present work. More interesting are Klein's mja5 and mja8 which were rejected by the base-composition gene-finder because, in both cases, ρ(CG) < 0.50. We note that both of these loci overlap putative protein-coding regions in *M.jannaschii*, one of which (mja5) has significant ($e < 0.002$) homology with methyl transferase and other proteins in *Methanosarcina barkeri*, *Methanothermobacter thermautotrophicus* and *Methanosarcina mazei*. On the other hand, Table 4 includes 13 candidate ncRNAs not predicted by Klein *et al.* and consequently not experimentally tested by them.

One intriguing aspect of Klein *et al.*'s work is their combination of a (G+C)% based HMM with a comparative genomics gene-finder (6). By using this combined approach, they can decrease the number of false positives that might be generated by using (G+C)% scanning alone. As mentioned above, we expect our gene-finder to have a lower false positive rate than Klein's since we use ρ(CG) in addition to (G+C)%. Nevertheless, combining a comparative genomics approach with our base-composition scanner should also be helpful in lowering the overall false-positive rate.

A different class of ncRNA gene-finders that can be compared with the present approach is represented by the comparative genomics methods (6–8). These powerful methods have found several ncRNAs in *E.coli* and show promise of being applicable to other species. Their principal limitation is the requirement of two—or preferably more—fully sequenced genomes of closely related species. RNAs that are not shared by related species will not be found by these methods. Moreover, it is not clear how well these methods will scale when applied to larger genomes with lower gene densities than *E.coli*.

It is interesting to speculate whether base-composition RNA-gene-scanning could have applicability in other genomes if used in combination with a structure-based RNA gene-finding approach such as that of Rivas *et al.* (6) or Eddy and Durbin (32). Since such structure-based RNA-gene-finders can be computationally demanding, it may be useful to have a rapid method to serve as an initial screen to eliminate part of the genomic background. For example, preliminary tests on short (1–2 MB) regions of the *C.elegans* genome identified a 5% (non-contiguous) subregion containing 80% of the GenBank-annotated tRNAs and rRNAs (data not shown). Though preliminary, these results suggest that in some genomes, such as *C.elegans*, base-composition scanning—though not sufficiently specific to identify ncRNAs by itself—may still be useful as a prescreening tool prior to the application of more discriminating, but computationally more demanding, RNA gene-finding programs.

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