Protein Coding Palindromes Are a Unique but Recurrent Feature in *Rickettsia*

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Rickettsia are unique in inserting in-frame a number of palindromic sequences within protein coding regions. In this study, we extensively analyzed repeated sequences in the genome of *Rickettsia conorii* and examined their locations in regard to coding versus noncoding regions. We identified 656 interspersed repeated sequences classified into 10 distinct families. Of the 10 families, three palindromic sequence families showed clear cases of insertions into open reading frames (ORFs). The location of those in-frame insertions appears to be always compatible with the encoded protein three-dimensional (3-D) fold and function. We provide evidence for a progressive loss of the palindromic property over time after the insertions. This comprehensive study of *Rickettsia* repeats confirms and extends our previous observations and further indicates a significant role of selfish DNAs in the creation and modification of proteins.

Interspersed repeated DNA sequences are usually confined in the intergenic regions of bacterial genomes. However, *Rickettsia* appears to be a unique exception in this respect. In our previous work, we identified evolutionarily related sequences of 50 amino acid residues dispersed in protein-coding regions of *Rickettsia conorii* and other *Rickettsia* (Ogata et al. 2000). The peptide segments showed no sequence similarity to known protein domains. On the other hand, the corresponding nucleotide sequences (~150 bases) showed imperfect palindromic (self-complementary) properties that resemble other bacterial intergenic repeats like IRU (Sharples and Lloyd 1990) and RSA (Bachellier et al. 1996). The repeats were designated as *Rickettsia* palindromic elements (RPEs). On the basis of the predicted locations of the inserts in the three-dimensional (3-D) folds of proteins, and on the observed transcript sizes, it is most likely that the repeat-derived peptide is expressed as part of the proteins encoded by those open reading frames (ORFs) (Ogata et al. 2000). Thus the RPE appears to have a unique capability to spread over the coding, as well as the noncoding, regions of the bacterial genome.

The completion of the genome sequence of *R. conorii* revealed a high density of repeated sequences in the genome (Ogata et al. 2001b). In this study, we systematically analyzed the repeat locations in regard to coding versus noncoding regions. Three different palindromic sequence families showed clear cases of insertions into ORFs. In addition, several palindromic sequences were identified within RNA coding genes. We also found that the palindromic property of the repeats has a tendency to be dimmed over time after their insertions in the genome.

RESULTS

We identified 656 interspersed repeated DNA sequences in the genome of *R. conorii*. On the basis of sequence similarity, the repeated sequences were classified into 10 distinct families (Table 1). There is no significant sequence similarity between the various repeat families. Their copy numbers range from 5 to 223. Nucleotide sequence alignments of the repeated sequences are shown in Figure 1. A coloring scheme is used to help visualize the predicted RNA secondary structures in the alignments. Of the 10 families, eight showed palindromic sequences with consensus sizes from 95 to 149 bases. Stable RNA secondary structures were predicted for most of the sequences in those eight families. The predicted secondary structures showed hairpin-like forms or variants with additional branched stems. However, the precise base-pairing pattern in the structures varied across, as well as within, the repeat families. The eight palindromic sequence families were named RPE-1 to RPE-8. The previously reported 44 RPEs (Ogata et al. 2000; Ogata et al. 2001b) were classified into the RPE-1 family, for which 11 additional copies have been identified in this study. The two remaining families are composed of shorter repeats (25 bases and 27 bases) showing no stable predicted secondary structure. They were designated as *Rickettsia* repeat-1 (RR-1) and repeat-2 (RR-2).

Exhaustive BLAST searches against sequence databases revealed that all of the 10 families except the RPE-6 are specific to *Rickettsia* species or limited to *R. conorii* (Table 1). The RPE-6 repeat contains two directly oriented RS3 core motifs (ATTCCC-N_8-GGGAAT) frequently found in *Neisseria* genomes (Haas and Meyer 1986). All the repeat families are relatively GC-rich (~40% of GC) compared with the average GC content of the entire genome (32%), with the exception of the AT-rich RPE-8 (22%). Size variation within family is large for six families (RPE-3 to RPE-8). More than 50% of the identified repeats are "partial" repeats for those RPEs. Size variation is relatively small for the other families (RPE-1 and RPE-2; RR-1 and RR-2), which are mostly composed of "full-length" copies (see Methods).

The analysis of the whole 656 repeat locations revealed a large number of insertions within ORFs for seven RPEs (RPE-1 to RPE-7) and a single occurrence for RR-1 (Table 1). Those cases include full-length repeat insertions within "annotated"

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Article and publication are at http://www.genome.org/cgi/doi/10.1101/gr.227602. Article published online before print in April 2002.

12:808–816 ©2002 by Cold Spring Harbor Laboratory Press ISSN 1088-9051/01 $5.00; www.genome.org

Table 1. Interspersed Palindromic Repeats and Short Repeats in *Rickettsia conorii*

Name	Total number	Size (bp)[a]	RNA 2-D structure	G + C	Full length repeat[b] Number	Full length repeat[b] Within gene	Partial repeat[b] Number	Partial repeat[b] Within gene	Distribution in other species	Number in *R. prowazekii*
Rickettsia palindromic elements (RPEs)										
RPE-1	52	141 (25–150)	hairpin	42%	45	24[c]	7	3	*Rickettsia* spp.	10
RPE-2	7	105 (76–105)	hairpin	43%	7	5	—	—	—	—
RPE-3	12	116 (28–122)	hairpin	40%	4	4	8	2	*Rickettsia* spp.	4[e]
RPE-4	94	95 (20–103)	hairpin	43%	19	2	75	11	*Rickettsia* spp.	15
RPE-5	55	115 (22–130)	hairpin	40%	26	2[d]	29	3	*Rickettsia* spp.	11[f]
RPE-6 (RS3-like)	168	136 (20–141)	clover leaf, Y-shape	39%	23	1	145	26	*Rickettsia* spp. *Neisseria* spp.	3
RPE-7	223	99 (20–136)	clover leaf, hairpin	43%	36	1	187	26	*Rickettsia* spp.	14
RPE-8	31	149 (30–172)	hairpin	22%	15	—	16	—	*Rickettsia* spp.	17
Rickettsia repeats (RRs)										
RR-1	5	27 (19–27)	—	49%	5	1	—	—	—	—
RR-2	9	25 (22–39)	—	47%	8	—	1	—	—	—

[a]Sizes of the consensus sequences. The lengths for the smallest and the largest copies are shown in parentheses.
[b]See text for the definition of the "full size" repeat and the "partial" repeats.
[c]One of the repeats is within the tmRNA gene (*ssrA*) of *R. conorii.*
[d]One of the repeats is within the M1 RNA gene (*rnpB*) of *R. conorii.*
[e]Three copies are within the ORFs (RP037, RP012, RP707) of *R. prowazekii.*
[f]One of the repeats is within the tmRNA gene of *R. prowazekii.*

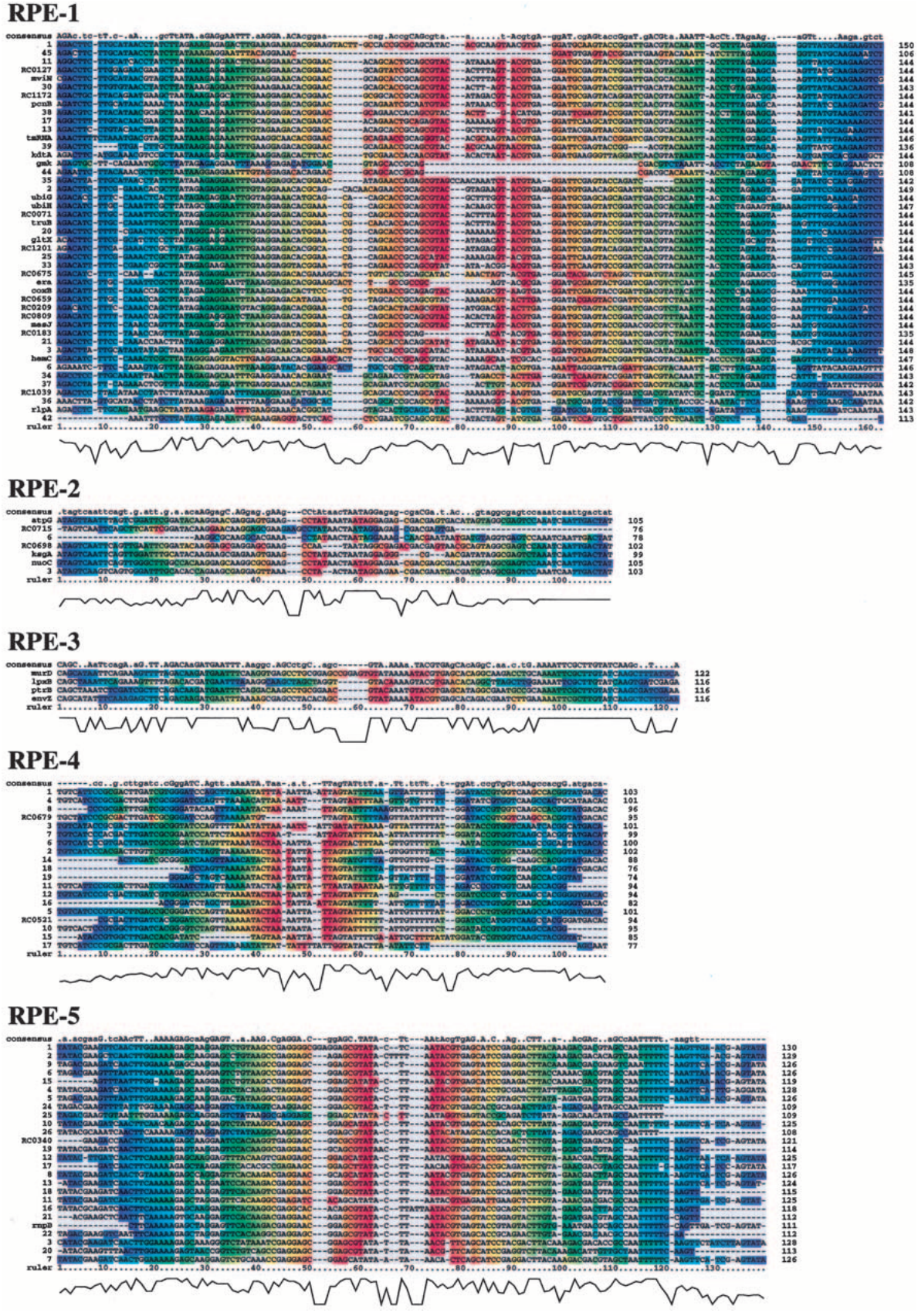
ORFs (ORFs with functions predicted by homology), as well as partial repeat insertions within ORFans (ORFs lacking similarity in other organisms). None of the insertions interrupts the reading frame of the host ORFs. Table 2 shows the 38 ORFs harboring the full-length repeats. Of the seven palindromic families found within ORFs, three families (RPE-1 to RPE-3) showed a number of full-length insertions into annotated ORFs. Most of those ORFs appear to be important for *R. conorii*, because they constitute parts of biological pathways or molecular complexes involving many other genes that are present in the *R. conorii* genome (Ogata et al. 2001b). However, there is no apparent functional relationship between the predicted functions of those altered ORFs.

By use of BLAST, we examined the occurrence of the homologous repeats in the *Rickettsia prowazekii* genome (Table 1). Interestingly, three copies of the RPE-3 were found within the ORFs of *R. prowazekii*: RP037 (putative O-sialoglycoprotein endopeptidase) and two ORFs of unknown functions (RP012 and RP707). Each of the three ORFs has a clear ortholog in *R. conorii*, which lacks the repeat insert. We previously reported nine cases of the RPE-1 within *R. prowazekii* ORFs (Ogata et al. 2000). In addition, a copy of an RS3-like element has been found in the N-terminal of the α subunit of DNA polymerase III (DnaE) in *Rickettsia felis* (Andersson and Andersson 1999). Thus, the insertions of palindromic sequences within ORFs seem to be a widespread phenomenon in different *Rickettsia* species.

Multiple alignments of the peptide sequences derived from the RPE-1 to RPE-3 are shown in Figure 2. Each of the three RPEs shows distinct peptide sequences with unbiased amino acid compositions. The peptide sequences are well aligned within the family and correspond to the same reading frame. A remarkable feature of those RPEs is the capability of occupying any site, even in the middle, along the primary sequences of the ORFs (Table 2). For instance, an RPE-1 sequence is located in the middle part of the *R. conorii* MesJ protein. The predicted protein secondary structure is a central α-helix for the RPE-1 and the RPE-3. In contrast, extended conformations (β-strands) were predicted at both extremities of the peptide sequences derived from the RPE-2. Those three peptide families may thus show different 3-D folds.

We then examined the insertion sites of the RPE-derived peptides with 3-D structure data for the homologs of the host proteins. Seven protein structures for RPE-1 insertions (Ogata et al. 2000), two for RPE-2 and two for RPE-3 (Table 2), were available for this analysis. In all cases, the insertion site corresponded to the solvent-exposed area of the proteins: mainly loops (nine cases) and occasionally short helices (one case) or beta strands (one case). Furthermore, none of the predicted insertions appeared to hinder known catalytic sites or protein/cofactor binding sites. Four cases corresponding to the ORFs with RPE-2 and RPE-3 insertions are shown in Figure 3.

We identified several RPEs within RNA genes. The tmRNA coding genes (*ssrA*) of *R. conorii* and *R. prowazekii* harbor palindromic sequences of different families (an RPE-1 for *R. conorii* and an RPE-5 for *R. prowazekii*). tmRNA is an RNA molecule present in all known bacterial genomes. Its function is to rescue the ribosome stalled on an mRNA (Muto et al. 1998). tmRNA is composed of a tRNA-like domain and an mRNA-like domain (Fig. 4a). The structure of the tmRNA genes from α-proteobacteria (Keiler et al. 2000), together with the locations of the RPE-1 and RPE-5, are shown in Figure 4, b and c. The two insertions of the palindromic sequences within the tmRNA genes were both located right after the CCA-(3′) bases of the acceptor arm, where alanine is added by alanyl-tRNA synthetase. We examined the transcription status from the *R. conorii* tmRNA gene by reverse transcriptase-polymerase chain reaction (RT-PCR). The RT-PCR product showed the expected size with the repeat insert, demonstrating transcription of RPE-1 with the rest of the genes. Because there is no detectable sequence similarity between the RPE-1 and the RPE-5, those two insertions must have occurred independently in the dif-



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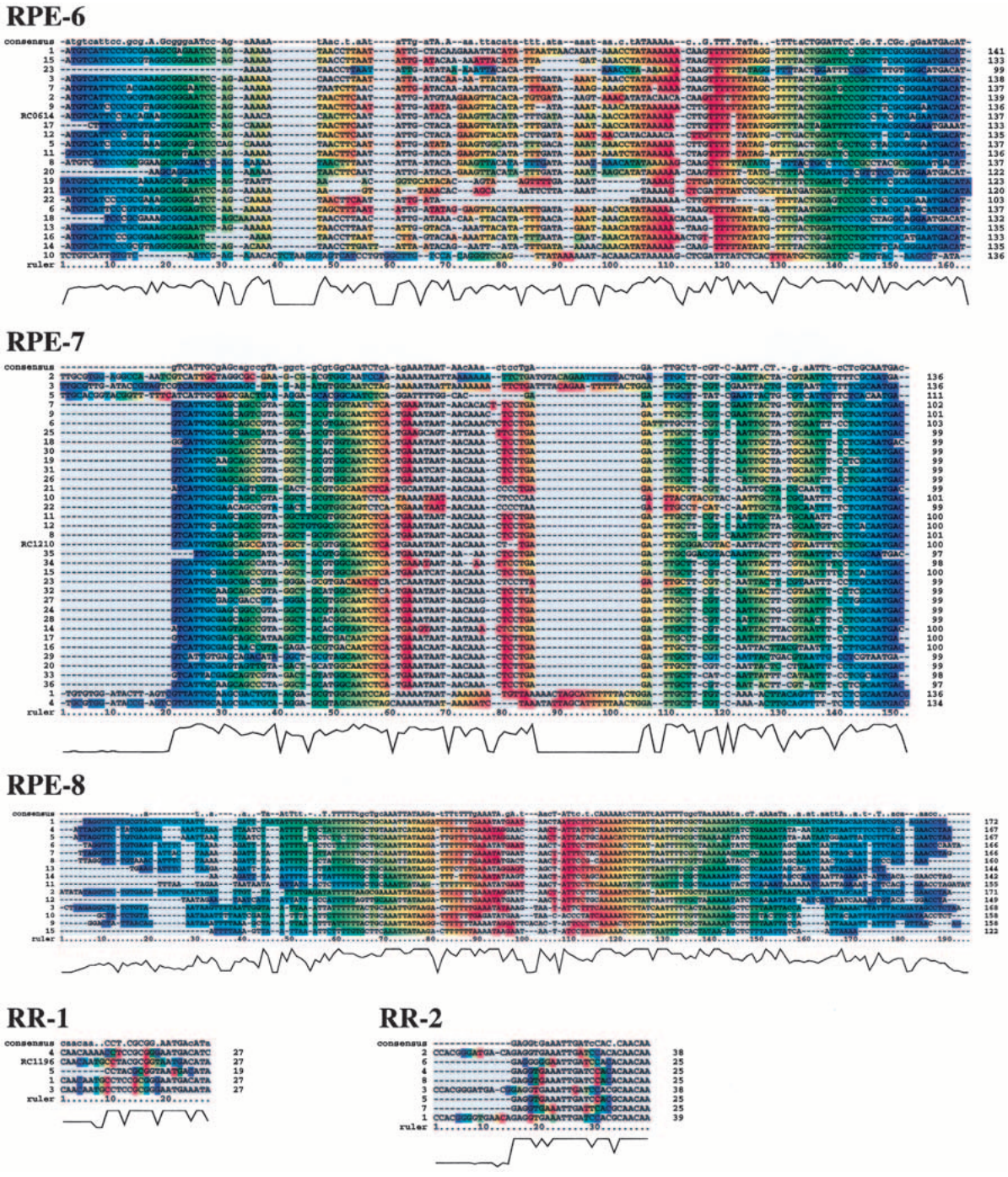

Figure 1 Nucleotide sequence alignments of the 10 repeat families identified in *Rickettsia conorii*. The coloring scheme is equivalent to the bracket representation of RNA secondary structure; the bases predicted to make base pairing are represented by the same color (instead of opening and closing brackets). The consensus sequences were derived from the aligned positions in which a single base is observed in at least 70% of the sequences (*lower* cases for 70%–90%; *upper* cases for ≥90% conservation). `ClustalX` alignment quality scores are shown at the bottom of the alignment.

ferent lineages of *Rickettsia*. Another case found in *R. conorii* is an RPE-1 within a ribozyme gene, *rmpB*, which encodes M1 RNA of the ribonuclease P. The insertion site corresponded to the P12 helix of the RNA secondary structure model of M1 RNA (Fig. 4d) (Brown et al. 1996). The P12 helix shows a highly variable sequence, and the helix is unlikely to involve functionally important tertiary interactions in vivo (Pomeranz Krummel and Altman 1999).

The palindromic property (hairpin-like secondary structure) of the RPEs is probably required for repeat insertion as

Table 2. Full Size Repeat Insertions within the *Rickettsia conorii* Genes

Gene name	Function	Phylogenetic distribution[a]	Size[b] (bp)	Location of repeat insertion[c]	Structure data[d]
RPE-1					
coxB (RC0555)	Cytochrome c oxidase polypeptide II	RPG--YOAE	945	16..159	1OCC/*Bos taurus*
era (RC0158)	GTP-binding protein	RPG-SYO-E	1017	10..144	1EGA/*Escherichia coli*
gltX (RC0966)	Glutamyl-tRNA synthetase	RPGCSYOAE	1539	1006..1149	1GLN/*Thermus thermophilus*
gmk (RC1194)	Guanylate kinase	RPGC-YO-E	687	19..126	1GKY/*Saccharomyces cerevisiae*
hemC (RC0706)	Porphobilinogen deaminase	RPGC-YOAE	1053	772..918	1PDA/*Escherichia coli*
kdtA (RC0118)	3-deoxy-D-manno-octulosonic-acid transferase	RP-C--O--	1392	147..290	
mesJ (RC0067)	Cell cycle protein MesJ	RPGCSYO--	1434	625..768	
mviN (RC0898)	Virulence factor MviN	RP-CSYO--	1665	6..149	
pcnB (RC0015)	Poly(A)polymerase	RPGCSYO-E	1308	120..263	
rlpA (RC0537)	Rare lipoprotein A precursor	RP--SYO--	960	33..175	
ssrA	tmRNA precursor	RPGCSYO--	474	78..223	
truB (RC0665)	tRNA pseudouridine 55 synthase	RPGCSYOAE	1035	784..927	
ubiG (RC0965)	3-demethylubiquinone-9 3-methyltransferase	RP------E	867	148..291	
uhiH (RC0848)	Ubiquinone biosynthesis protein	RP---Y--E	1293	31..177	
RC1039	Split gene of mannose-1-phosphate guanylyltransferase	-P---YOA-	627	9..151	
RC0071	Unknown function	RP---YO-E	1218	985..1128	
RC0127	Unknown function	---------	222	42..185	
RC0183	Unknown function	---------	1158	706..840	
RC0209	Unknown function	R--------	279	22..165	
RC0659	Unknown function	R--------	582	31..174	
RC0675	Unknown function	---------	225	18..162	
RC0809	Unknown function	RPGCSYO--	735	349..492	
RC1172	Unknown function	---------	345	18..161	
RC1201	Unknown function	---------	240	100..243	
RPE-2					
atpG (RC1236)	ATP synthase γ chain	RPG--YO-E	969	622..726	1H8E/*Bos taurus*
ksgA (RC1022)	Dimethyladenosine transferase	RPGCSYOAE	945	370..468	1YUB/*Streptococcus pneumoniae*
nuoC (RC0483)	NADH dehydrogenase I chain C	RPG--YOAE	726	199..303	
RC0698	Unknown function	R--------	1002	100..201	
RC0715	Unknown function	R--------	753	299..374	
RPE-3					
envZ (RC0592)	Osmolarity sensor protein EnvZ	RP-------	1437	34..149	
lpxB (RC0440)	Lipid-A-disaccharide synthase	RP-C-YO--	1338	1138..1253	
murD (RC0560)	UDP-N-acetylmuramoylalanine-D-glutamate ligase	RPGCSYO--	1500	796..917	1E0D/*Escherichia coli*
ptrB (RC0377)	Protease II	RPG------	2187	526..641	1QFM/*Sus scrofa* (pig)
RPE-4					
RC0521	Unknown function	---------	336	29..122	
RC0679	Unknown function	---------	1806	243..337	
RPE-5					
RC0340	Unknown function	---------	171	45..165	
rnpB	RNA subunit (M1 RNA) of ribonuclease P	RPGCSYOAE	458	118..228	
RPE-6					
RC0614	Unknown function	---------	387	198..334	
RPE-7					
RC1210	Unknown function	---------	303	30..129	
RR-1					
RC1196	Unknown function	---------	180	9..35	

[a]Abbreviations for the organism groups are as follows. R: *Rickettsia* (*Rickettsia prowazekii*); P: Proteobacteria (*Escherichia coli* K-12, *Haemoplilus influenzae, Xylella fastidiosa, Vibrio cholerae, Pseudomonas aeruginosa, Buchnera* sp., *Neisseria meningitidis* serogroup A and B, *Helicobacter pylori* 26695 and J99, *Campylobacter jejuni*); G: Gram positive bacteria (*Bacillus subtilis, Bacillus halodurans, Mycoplasma genitalium, Mycoplasma pneumoniae, Ureaplasma urealyticum, Mycobacterium tuberculosis;* C: *Chlamydia* (*Chlamydia trachomatis, Chlamydia muridarum, Chlamydia pneumonia* CWL029, AR39 and J138); S: Spirochete (*Borrelia burgdorferi, Treponema pallidum*); Y: Cyanobacteria (*Synechocystis*); O: Other bacteria (*Deinococcus radiodurans, Aquifex aeolicus, Thermotoga maritima*); A: Archaea (*Methanococcus jannaschii, Methanobacterium thermoautotrophicum, Archaeoglobus fulgidus, Halobacterium* sp., *Thermoplasma acidophilum, Pyrococcus horikoshii; Pyrococcus abyssi, Aeropyrum pernix*); E: Eukaryotes (*Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster*). When there is no homolog within an organism group, '-' replaces the organism abbreviation.

[b]Gene size without stop codon.

[c]The repeat location is indicated by the base position within the gene.

[d]Protein Data Bank identifiers and the organism names for the available structure data.

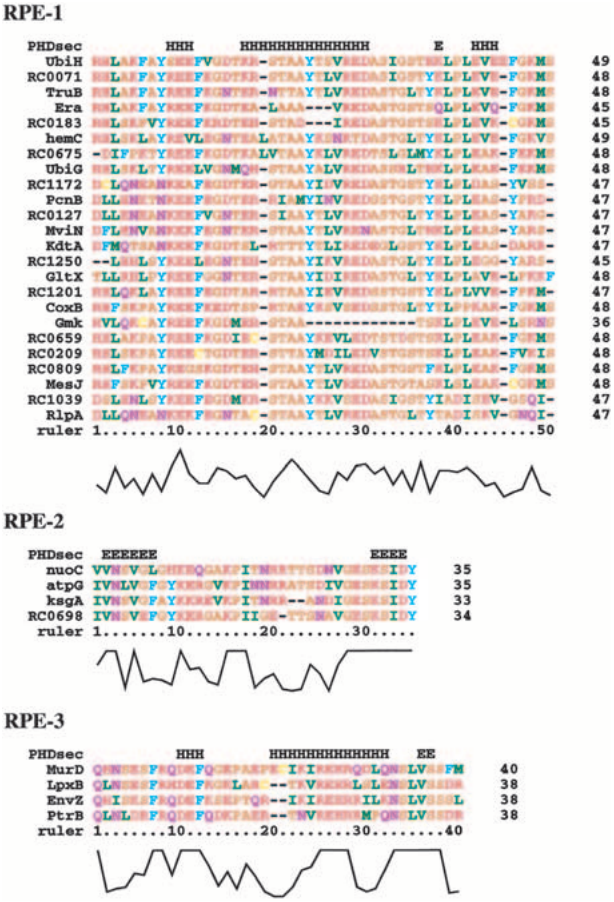

Figure 2 Multiple sequence alignments for the amino acid sequences translated from the palindromic sequences (RPE-1, RPE-2, and RPE-3). Amino acid residues are colored as follows: blue for F, W, and Y; yellow for C; orange for A, G, P, S, and T; green for I, L, M, and V; red for D, E, H, K, and R; purple for N and Q. The letters 'H' and 'E' in the first line of each alignment represent the predicted α-helices and β-strands, respectively. `ClustalX` alignment quality scores are shown at the bottom of the alignment.

suspected for IRU (Sharples and Lloyd 1990) and RSA (Bachellier et al. 1996). Alternatively, the hairpin structures might have an important function for *Rickettsia*. In the former case, the hairpin-like structure might lose its utility after insertions and could disappear over time. In the latter case, nucleotide secondary structures should be conserved despite sequence changes (as in ribosomal RNAs). To investigate the significance of the palindromic property of the RPEs, we computed the minimum free energy for every sequence of the RPE-1 to RPE-8 and obtained the relevant Z-score (see Methods). If we take Z-score > 2 (P-value < .0423) as a threshold, the energy values for 10/45 sequences (22%) of the RPE-1 failed to be significant. Such energy values below the threshold were also observed for the RPE-2 (4/7; 57%), the RPE-5 (6/20; 23%), and the RPE-8 (3/15; 20%). This result indicates that the palindromic properties of some repeats are unlikely to be constrained after their insertions. In Figure 5, the Z-score of the RPE-1 is plotted against the sequence divergence D, the average sequence difference against the other sequences of the RPE-1. The pair of most similar sequences, which might correspond to most recent inserts, showed very high Z-scores (Z = 8.30 and 8.24). The two sequences were identified in the *truB* gene and in RC0071. The 144 bases sequences are 94.4% identical with each other. There is also a global tendency for the better conserved sequences to show higher stabilities of the RNA secondary structures (the correlation coefficient is $R = -0.74$; $P < .005$). This decay of palindromic property indicates the absence of structural constraints on the repeats after their insertions. The lack of significant differences in the structural stability between the coding and the noncoding repeats also argues against a specific role of the palindromic structures at the transcription and translation levels.

DISCUSSION

In this study, we identified 10 families of repeated sequences in the genome of *R. conorii* and examined their locations in regard to coding versus intergenic regions. Three palindromic sequence families (RPE-1, RPE-2, and RPE-3) showed clear cases of insertions within predicted coding regions, and eight families in total (RPE-1 to RPE-7 plus RR-1) showed insertions within coding regions including ORFans. Therefore, the surprising mechanism of repeat insertion within protein coding regions initially described for RPE-1 (Ogata et al. 2000) applies to many other repeat families in *Rickettsia*.

The analysis of the locations and the sequences of the repeat-derived peptides (RPE-1, RPE-2, and RPE-3) reinforced our previous observations (Ogata et al. 2000). First, they are inserted into ORFs with only one reading frame of six possibilities, as indicated by the aligned sequences of the repeat-derived peptides (Fig. 2). Second, there are no clear functional links between the ORFs harboring the repeats. Third, the insertion sites of the repeats vary along the primary sequence of the ORFs but always appear compatible with the preexisting protein folds.

This study revealed two additional aspects. First, the predicted protein secondary structures for the three RPEs (RPE-1 to RPE-3) correspond to two different conformations. α-Helices were predicted for the RPE-1 and RPE-3, whereas β-strands were predicted for the RPE-2. Thus, the two regular conformations in protein structure (α-helix and β-strand) could occur from repeat insertions. However, the possibility is not ruled out that those peptides are "neutral" and might adapt variable conformations in response to the surrounding structural environment at the insertions sites. Circular dichroism spectroscopy failed to show any property of the regular conformation for synthetic peptides (~50 amino acids) corresponding to the RPE-1 (C. Abergel, unpublished data). Four repeat-containing proteins have been expressed in *Escherichia coli* (V. Monchois et al., in prep.), and experiments are in progress to determine the structural properties of the repeat-derived peptides within these proteins.

Second, we showed that some copies of the RPEs do not exhibit a significant palindromic structure. Because the pair of most similar RPE-1 sequences correspond to highly stable hairpins, it is plausible that this secondary structure is a feature of the original copies that are mobile within the genome. The structural property might then be lost after the insertion regardless of the site of the genome (coding or noncoding), as the initial repeat continued to diverge in both sequence and structure. However, the possibility is not ruled out that some of the repeats have been recruited for host cellular functions (Gilson et al. 1984; Gilson et al. 1986a; Gilson et al. 1986b; Sharples and Lloyd 1990) or recombination (Bi and Liu 1996; Oggioni and Claverys 1999; Shyamala et al. 1990; van der Ende et al. 1999), as already suggested for other bacterial repeats.

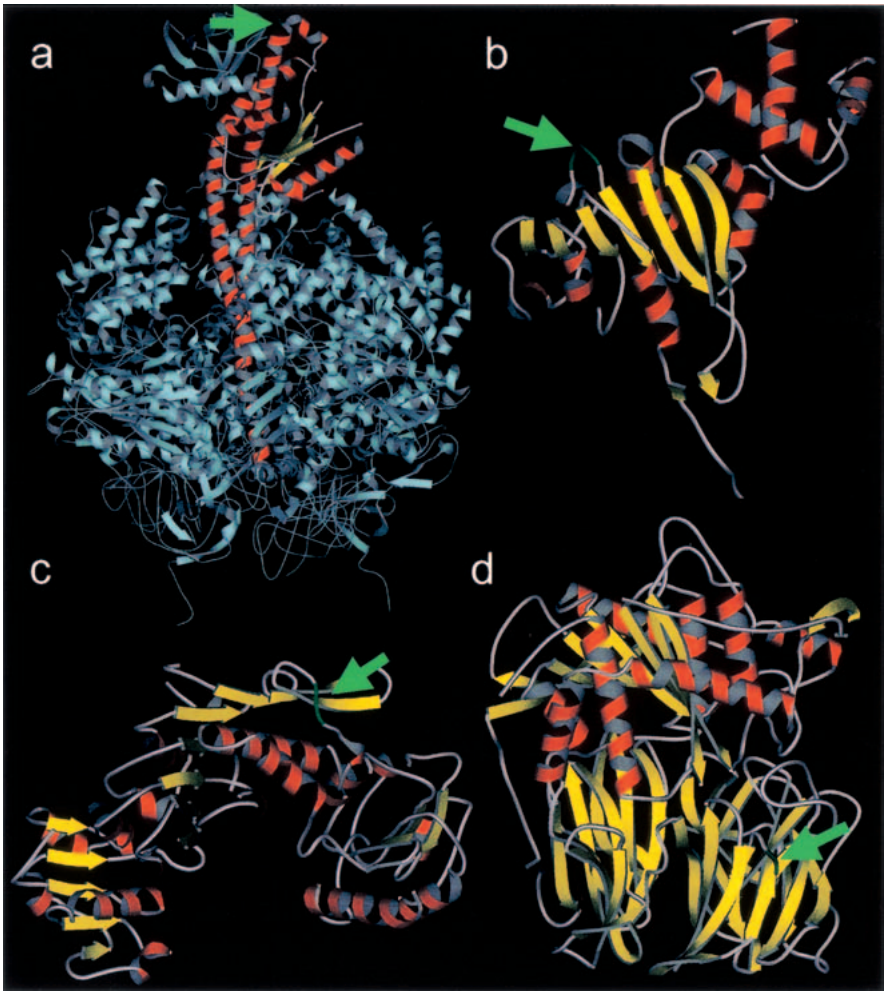

Figure 3 Predicted insertion sites of the RPE-2 (*a, b*) and RPE-3 (*c, d*). Green segments labeled by the green arrows in the reference structures indicate the insertion sites. (*a*) Bovine mitochondrial ATP synthase. γ chain is shown in red. (*b*) *Streptococcus pneumoniae* rRNA methyltransferase used as a reference for the *R. conorii* dimethyladonosine transferase. (*c*) *E. coli* UDP-N-acetylmuramoylalanine–D-glutamate ligase. (*d*) Pig prolyl oligopeptidase used as a reference for the *R. conorii* protease II. Seven cases corresponding to the predicted insertions sites for the RPE-1 have been previously reported (Ogata et al. 2000). The images are generated with `MolScript` (http://www.avatar.se/molscript/).

Proteins contain structurally flexible regions, usually corresponding to surface loops. Such loops are known to be tolerant of insertions of individual amino acids or peptides. For instance, insertions of peptides between 7 and 17 residues into a loop of the chymotrypsin inhibitor-2 (64 amino acids) have little effect on the stability and the folding rate (Ladurner and Fersht 1997). This physical flexibility parallels the evolutionary flexibility of protein sequences. Available sequence and structure data indicate a high preference of insertions and deletions within loops (Pascarella and Argos 1992). However, most (99%) of the accepted insertions and deletions are shorter than 10 amino acid residues. In contrast, the palindromic repeats described in this study could contribute to insertions up to ~50 residues. Repeat insertion can freely occur within the 20% of *R. conorii* genome corresponding to the noncoding regions. If one accepts that surface loops account for a quarter of every protein sequence (Wootton 1994), another 20% of the genome (from the coding moiety) is available for additional repeat insertions. RPEs appear to invade both of the two genomic regions.

The mechanism by which the bacterial palindromic sequences of the size of RPEs spread within genomes is not known (Bachellier et al. 1999). However, the coincidence of the two insertion sites of the palindromic sequences in the tmRNA genes (*ssrA*) of *Rickettsia* is intriguing. The upstream sequences of those insertion sites are highly similar to bacteriophage attachment site (*att*) (Kirby et al. 1994). The homologous sites of the other tmRNA genes and tRNA genes have been known to harbor bacteriophages, retron phages, and pathogenicity islands in other bacteria (Billington et al. 1999; Haring et al. 1995; Inouye et al. 1991; Julio et al. 2000; Karaolis et al. 1998; Kirby et al. 1994; Pierson and Kahn 1987). Such retron phages and pathogenicity islands are supposed to be integrated by use of the integrases of phages. Some of the RPEs might have used a similar mechanism.

We have proposed that RPEs are selfish DNA elements that can break the barrier of genetic material between coding and noncoding sequences (Ogata et al. 2000). Recursive insertions of such selfish DNAs might provide the initial genetic material for rather "neutral" protein segments, which could then later evolve to create new functions (Dwyer 2001; Ogata et al. 2001a). The genomes of *Rickettsia* show by far the highest number of occurrence of such insertions, even if a few instances have been reported in other bacteria. For instance, a partial copy of RSA was found in the C-terminal of a hypothetical ORF of 99 amino acids in *Salmonella typhimurium* (Bachellier et al. 1996). Recently another case has been reported in *Sinorhizobium meliloti*. The DNA helicase II (UvrD) of this legume symbiont has a 47 amino acid residues insert encoded by a palindromic DNA sequence (motif C) (Capela et al. 2001). The ongoing accumulation of more bacterial genome sequences should lead to better appreciation of the importance of the phenomenon of repeat insertions in the origin and evolution of proteins.

METHODS

The genomic sequence and annotation data for *R. conorii* are available at RicBase (http://igs-server.cnrs-mrs.fr/RicBase) and NCBI GenBank (http://www.ncbi.nlm.nih.gov/; accession no. AE006914). Other complete genomes including those used in Table 2 were obtained from KEGG (Kanehisa and Goto 2000). Database searches were performed with the NCBI `BLAST` package (Altschul et al. 1997) against the complete genomes as well as the NCBI nonredundant sequence database.

Repeated DNA sequences of *R. conorii* were initially identified on the basis of the self-comparison of the genomic DNA by BLASTN (E-value < 10^{-4}). The BLAST result was then analyzed to delineate the left and right edges of the repeated sequences with the repeat identification program Mocca (Notredame 2001). Some trivial repeats such as tRNAs or paralogous ORFs were removed from the dataset a posteriori. A complete list of the repeats described in this paper is available at RicBase.

The "full-length" repeats were defined as the sequences with lengths within 70% to 100% of the longest repeat of the family. The remaining shorter sequences were defined as "partial" repeats. The nucleotide sequence alignments and the consensus sequences in Figure 1 were constructed from the full-length repeats with T-Coffee (Notredame et al. 1998) and ClustalX (Thompson et al. 1997). Sequence divergence D used in Figure 5 was defined as the average sequence difference against the other sequences of the family. The definition of D does not take into account nucleotide secondary structures. In the computation of D, the positions with gaps in the pairwise alignments were omitted.

The minimum free energy and the corresponding RNA secondary structures were computed with the Vienna package at http://www.tbi.univie.ac.at/~ivo/RNA/ (Hofacker et al. 1994). The minimum free energy value was then converted to Z-score. To compute Z-score, every sequence was randomly shuffled 30 times, from which the mean and the standard deviation values were computed. We used an approximation by the extreme value distribution (Gumbel 1958) to obtain the relevant *P* value. Protein secondary structures were predicted with PHDsec at http://dodo.cpmc.columbia.edu/predictprotein/ (Rost and Sander 1994).

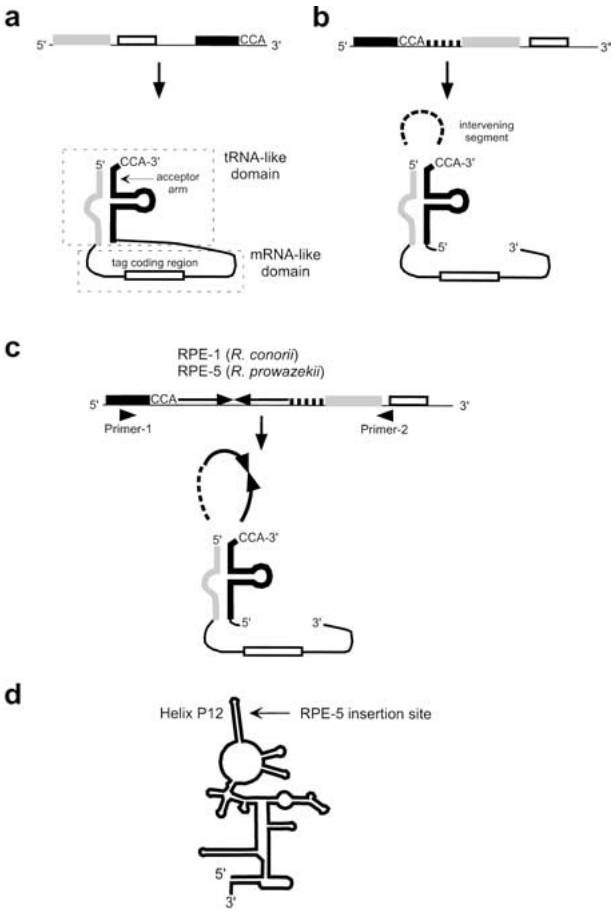

Figure 4 RPEs found within the RNA genes. (*a*) The standard form of the tmRNA genes observed in most bacterial species. (*b*) The permutated form of the tmRNA gene observed in α-proteobacteria (Keiler et al. 2000). (*c*) Locations of the RPE-1 and RPE-5 inserted in the tmRNA genes of *Rickettsia*. Approximate locations of the primers are indicated by triangles. (*d*) The secondary structure model of the M1 RNA and the insertion site of the RPE-5 in *R. conorii*.

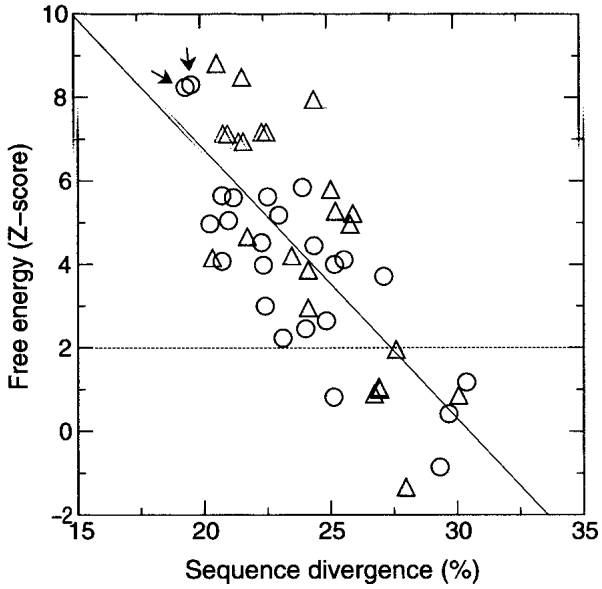

Figure 5 The minimum free energy of the predicted RNA secondary structures of the RPE-1 plotted against sequence divergence. The energy values are scaled into Z-score. Circles correspond to the repeats in coding regions. Triangles correspond to the repeats in noncoding regions. The arrows point to the two most similar sequences.

The following protein structure data were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/): Bovine mitochondrial ATP synthase F_1 domain (1H8E); *Streptococcus pneumoniae* rRNA methyltransferase (1YUB); *E. coli* UDP-N-acetylmuramoylalanine–D-glutamate ligase (1E0D); and Pig prolyl oligopeptidase (1QFM). The rRNA methyltransferase was used as a reference for *R. conorii* dimethyladenosine transferase (KsgA); they belong to the rRNA adenine N-6-methyltransferase family. The prolyl oligopeptidase was used as a reference structure for *R. conorii* protease II (PtrB); they both belong to the prolyl oligopeptidase family.

The presence of the RPE-1 in the transcript from the tmRNA gene of *R. conorii* was assessed by RT-PCR by use of a primer pair, P1 (5′-TAA TTT AGA ATA GAG GTT GCG GAC T-3′) and P2 (5′-CGT TTG CGT TTC TTT GTT TT-3′), designed to be specific to the target gene. The expected size of the RT-PCR product was 311 bp including the RPE-1 (146 bp). For RNA extraction, a suspension of fresh *R. conorii* strain Malish (seven) was adjusted to 10^8/mL, and bacteria were separated from cells with a sucrose gradient. RNA extraction from bacteria was then performed with the RN easy Midi kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. RT-PCR was performed on the resulting RNA with the One-step RT-PCR kit (Qiagen) following the manufacturer's instructions. Reverse transcription and amplification were performed in PTC-200 thermocyclers (MJ Research, Watertown, USA) with 40 PCR cycles and an annealing temperature of 50°C. RT-PCR products were run in 1% agarose gels, stained with ethidium bromide, and revealed on a UV box. Following the RT-PCR assay, we performed a PCR assay with the same primers on the RNA extract with the Elongase polymerase

(Life Technologies, Cergy Pontoise, France). The PCR assay was negative, thus verifying the absence of contaminating DNA in the RNA. The RT-PCR product was sequenced with the same primers with the d-Rhodamine terminator cycle-sequencing ready reaction kit (PE Applied Biosystems, Les Ulis, France) and an ABI-PRISM 3100 automated DNA sequencer (PE Applied Biosystems), as recommended by the manufacturer. An RT-PCR product of 311 bp was obtained from the *R. conorii* RNA. The sequence of the RT-PCR product was 100% identical to the genomic sequence of the *R. conorii* tmRNA gene containing the RPE-1.

ACKNOWLEDGMENTS

We thank Professors Philippe Derreumaux and Didier Raoult for helpful discussions.

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Received December 13, 2001; accepted in revised form March 6, 2002.