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The Nucleotide Sequence of the Entire Ribosomal DNA Operon and the Structure of the Large Subunit rRNA of *Giardia muris*

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Summary. The total nucleotide sequence of the rDNA of *Giardia muris*, an intestinal protozoan parasite of rodents, has been determined. The repeat unit is 7668 basepairs (bp) in size and consists of a spacer of 3314 bp, a small-subunit rRNA (SSU-rRNA) gene of 1429, and a large-subunit rRNA (LSU-rRNA) gene of 2698 bp. The spacer contains long direct repeats and is heterogeneous in size. The LSU-rRNA of *G. muris* was compared to that of the human intestinal parasite *Giardia duodenalis*, to the bird parasite *Giardia ardeae*, and to that of *Escherichia coli*. The LSU-rRNA has a size comparable to the 23S rRNA of *E. coli* but shows structural features typical for eukaryotes. Some variable regions are typically small and account for the overall smaller size of this rRNA. The structure of the *G. muris* LSU-rRNA is similar to that of the other *Giardia* rRNA, but each rRNA has characteristic features residing in a number of variable regions.

Key words: *Giardia muris* — Protozoan parasite — Ribosomal rRNA genes — Ribosomal DNA sequence — Large-Subunit rRNA — Sequence comparison

Introduction

The majority of eukaryotes studied to date carry a set of tandemly repeated units containing the ribosomal RNA (rRNA) genes which consist of a nontranscribed spacer, a transcribed spacer, the small-subunit rRNA (SSU-rRNA) gene, an internal transcribed spacer, the 5.8S rRNA gene, a second internal transcribed spacer, and the large-subunit rRNA (LSU-rRNA) gene (Gerbi 1985). Variations on this theme include the size of the nontranscribed spacer, which can be either uniform or non-uniform in size within a species, and the size and sequence of the variable regions in the mature rRNA genes.

The SSU-rRNAs have proven useful in providing a phylogenetic framework to study taxonomic segregation of organisms (Woese 1987). Based on this criterion, the protozoan parasite *Giardia duodenalis* (synonymous for *G. lamblia* and *G. intestinalis*) has been described as representing the earliest-diverging lineage in the eukaryotic line of descent (Sogin et al. 1989) The *Giardia* rRNA genes are unusual in several respects: not only is the entire transcription unit small, but also the SSU-rRNA gene is small and resembles in size prokaryotic rDNA more closely than eukaryotic rDNA. However, the G + C content of the rRNA genes of *G. duodenalis* is high (75%), which could make comparison with other rRNA genes with lower G + C content problematic. *Giardia duodenalis* is only one of five known species of *Giardia*. The others are *G. muris*, *G. ardeae*, *G. psittaci*, and *G. agilis* (Filice 1952; Erlandsen and Bemrick 1987; Erlandsen et al. 1990). One of these species, *G. muris*, has a rDNA repeat unit that is larger and has a different restric-

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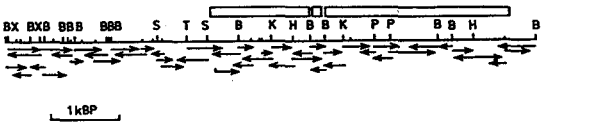


Fig. 1. The nucleotide sequence of *Giardia muris* rDNA. The RNA-like DNA strand of the longest rDNA is given corresponding to the map. (See insert.) The positions of the 5′ and 3′ ends of the SSU-rRNA (numbers 1 and 2) and 5.8S rRNA (numbers 3 and 4) genes are indicated with numbered arrowheads. Here, only the first 23 and last 37 bases of the LSU-rDNA are indicated with arrowheads (5, 6). The sequence of a shorter spacer-specific Sau3AI fragment, position 2139–2901, is aligned with and written under the DNA sequence; the identical base sequences are indicated by dots (.) under the bases, and deletions are indicated by asterisks (*). The positions of a number of direct repeats are indicated by underlining the beginning of each repeated sequence with a short arrow (→). The various restriction enzyme sites, corresponding to the map, are indicated by underlining. The closed arrows, position 128–973, indicate an open reading frame for 281 amino acids. Insert: physical and genetic map of the *G. muris* rDNA. The positions of the rRNA genes are indicated by boxes over the restriction enzyme map; from left to right the boxes represent SSU-rDNA, 5.8S rDNA, and LSU-rDNA. The arrows indicate the direction and extent of DNA sequence analysis. An oligonucleotide, used to supplement the sequence analysis, is indicated at the 5′ end of the SSU-rDNA as an upright bar on an arrow and has the sequence 5′GATCCTGCCGGAG3′. Restriction enzyme sites: BamHI (B); HindIII (H); KpnI (K); PvuII (P); Sau3AI (S); TaqI (T); XhoI (X); the short bars without letters are the SmaI sites.

tion enzyme recognition pattern than *G. duodenalis* (van Keulen et al. 1991a). The rDNA of *G. muris* also has a much lower G + C content (61.9%) than that of *G. duodenalis* (Boothroyd et al. 1987). To provide a basis for further analysis of the taxonomic and evolutionary position of the genus *Giardia*, the DNA sequence of the entire rDNA operon of *Giardia muris* was determined from available clones. This report will focus on the spacer and LSU-rRNA gene and its RNA structure. A comparison between the SSU-rRNA of *G. muris*, *G. duodenalis*, and *G. ardeae* and its implication in phylogeny will be presented in a later publication.

Materials and Methods

Isolation of DNA. Giardia muris trophozoites were obtained from cysts isolated from CF1 mice by standard methods (Robert-Thomson et al. 1976; Sauch 1984). Trophozoites were harvested from *G. muris* cysts which were induced to excyst as previously described (Campbell et al. 1990). Total genomic DNA was isolated according to a standard procedure described previously (van Keulen et al. 1985). Plasmid DNA was isolated by the alkaline minipreparation procedure of Birnboim (1983) modified by the use of ammonium acetate for the precipitation of the genomic DNA complex (Bird and Wu 1989).

Southern Blot Analysis. Total genomic DNA was digested (4 μg per digestion) with various restriction enzymes and fractionated according to size on 1% agarose gels. The gels were stained with ethidium bromide, photographed, and prepared for Southern transfer to nitrocellulose filters according to standard procedures (Maniatis et al. 1982).

All cloned DNAs used as probes were digested with the appropriate restriction enzymes and separated from vector DNA by agarose gel electrophoresis. The DNA fragments were purified by the freeze-phenol procedure (Benson 1984). Nick-translations and hybridizations were carried out as described previously (Campbell et al. 1990).

DNA Sequence Analysis. The appropriate DNA restriction enzyme fragments were size fractionated, purified, and cloned in M13mp18 or mp19 vector DNA with JM 101 or JM 109 as host strains (Messing 1983). For the single-stranded DNA isolations and the sequencing reactions, the protocols supplied with the Sequenase and TAQuence kits were used (United States Biochemical Corp., Cleveland, OH). Areas with extreme band compressions were analyzed by using dITP with Sequenase or 7-deaza-dGTP with TAQuence in the reaction labeling mixture. When necessary the standard 8% acrylamide/8 M urea sequencing gels were substituted for 8% acrylamide/8 M urea/20% formamide gels. Most of the sequence was determined by analysis of both strands; in small remaining sections where this could not be done the sequence analysis was repeated using the two different enzymes. The oligonucleotide used as internal primer for sequence analysis was synthesized in the microchemical facility at the University of Minnesota and had the sequence 5'GATCCT-GCCGGAC3'. The sequence data were analyzed with the DNASTAR sequence programs (DNASTAR Inc., Madison, WI). The sequence of the LSU-rRNA of *G. muris* was compared to those from *G. duodenalis* (Healey et al. 1990), *G. ardeae* (van Keulen et al. 1991b), *Escherichia coli* (Brosius et al. 1980), and *Halobacterium halobium* Mankin and Kagramanova 1986). Sequence similarity was determined by the DNASTAR alignment program, based on the procedure of Wilbur and Lipman (1983) with a K-tuple size of 5, range of 20, and gap penalty of 6. Sequence alignment was performed by aligning the sequences according to the procedure of Woese et al. (1983).

Secondary Structure. The secondary structure diagrams were drawn in a format based on that of *E. coli* 23S rRNA model, which may be regarded as the standard or prototype structure (Gutell et al. 1990). The figures were laid out with the assistance of a new RNA graphics program, XRNA, developed by Bryn Weiser (unpublished). Identification of some of the variable regions and estimates of their sizes were based on the compendium of 23S-like rRNAs by Gutell et al. (1990).

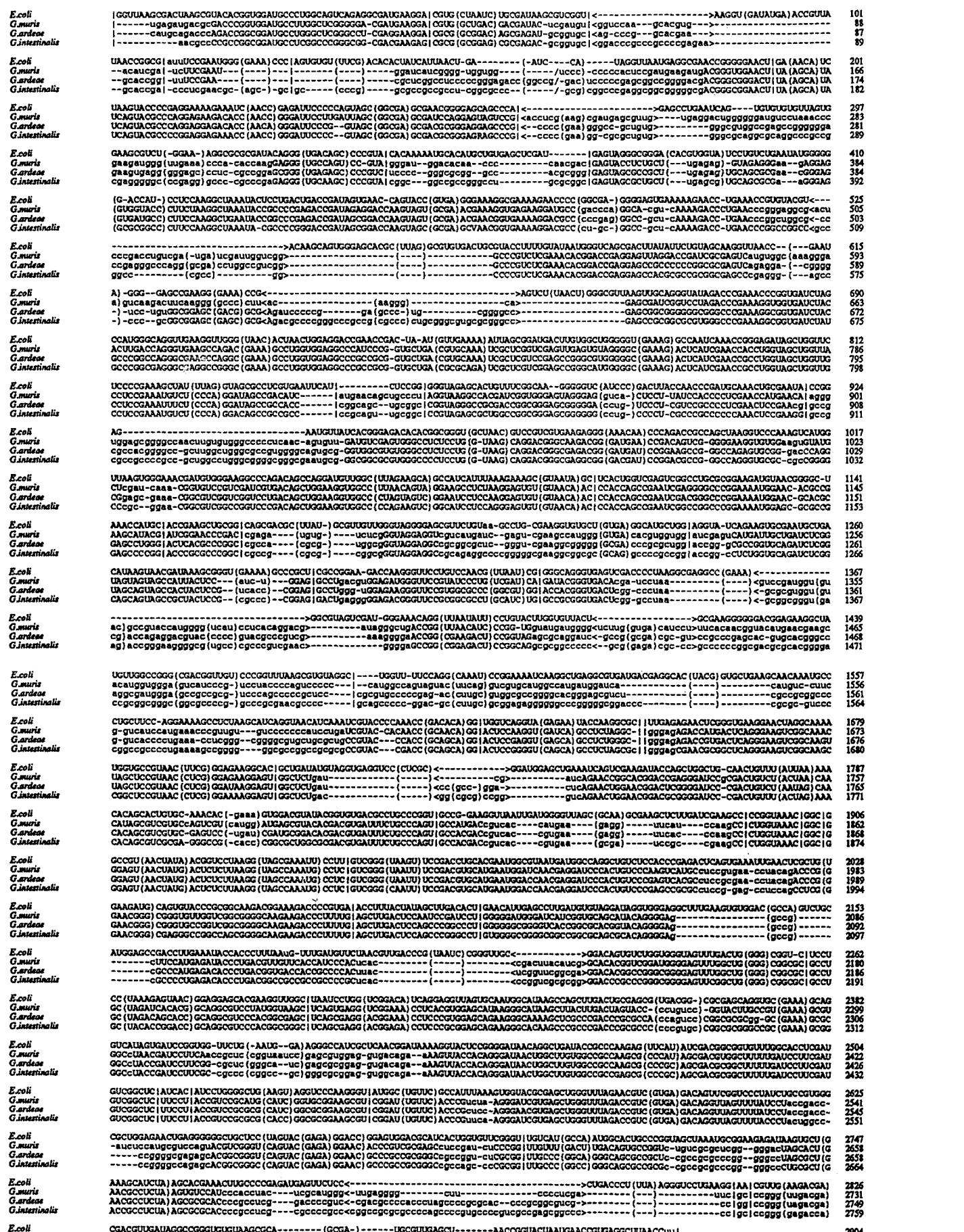
Results

Structure of the rDNA Operon and Spacer DNA

The rDNA repeat of *G. muris* contains two HindIII and two KpnI sites (Fig. 1). Cloning of the operon was performed by isolation of the two HindIII and three KpnI fragments from genomic DNA digests and insertion in plasmid vectors. The clones consist of HindIII fragments of 5.2-kilobasepair (kbp) (pGmr5.2) and 2.55 kbp (pGmr2.5) and of KpnI fragments of 6.5-kbp (pGmr6.5), 6.2 kbp (pGmr6.2), and 1.15 kbp (pGmr1.15). Digestion of the clones pGmr6.5 and pGmr6.2 with BamHI revealed that the size difference resided in 1.8- and 1.5-kbp BamHI fragments, respectively, which hybridized to SSU-rDNA probes (not shown). The size difference was caused by the presence of differently sized Sau3AI fragments in the various clones which are located 5′ to the SSU-rRNA gene (Fig. 1). Since differently sized clones could be artifacts from extension/deletion of the cloned DNAs in *E. coli* due to repeated sequences, the possible presence of variously sized BamHI fragments in genomic DNA was determined by hybridization of nick-translated Sau3AI fragment to size-fractionated restriction enzyme digests of *G. muris* genomic DNA. Indeed, four bands hybridized to the Sau3AI fragment, indicating that there were several differently sized spacer DNAs present. These same bands also hybridized to a SSU-rDNA probe (data not shown).

The restriction enzyme map and entire sequence

Fig. 2. Sequence alignment of *G. muris*, *G. ardeae*, *G. duodenalis* LSU-rRNA with *E. coli* 23SrRNA. The sequence of the LSU-rRNA of *G. muris* was aligned with the sequence of *G. ardeae* (van Keulen et al. 1991b), with *G. duodenalis* from position 856–3745 of the sequence published by Healey et al. (1990) for *G. intestinalis* except that a third C was inserted after position 3500 (unpublished observation) and with *E. coli* (Brosius et al. 1980). The dashes (-) indicate spaces created in the sequence for optimal alignment.


E.coli
G.muris
G.ardeae
G.intestinalis

for the SSU-rDNA, 5.8S rDNA, and spacer DNA and part of the LSU-rDNA of *G. muris* rDNA is presented in Fig. 1. The position of the LSU-rDNA is indicated by the first 23 and last 37 nucleotides. The entire sequence of the LSU-rDNA is shown separately in Figs. 2 and 3. The sequence of one of the shorter Sau3AI fragments in the spacer is indicated in Fig. 1, where it is aligned with the sequence of the same region corresponding to the largest Sau3AI fragment found. The observed size difference is obviously the result of variation in the number of repeated sequences in the spacer DNA. These repeats are indicated in the figure. The boundaries of the mature rRNAs that were determined previously (van Keulen et al. 1991a) are indicated by arrowheads (Fig. 1). The exact position of the 3′ end of the LSU-rRNA is the least certain.

The entire operon is 7668 nucleotide (nt) long and contains a SSU-rRNA gene of 1429 nt and a LSU-rRNA gene of approximately 2698 nt. The overall G + C content of *G. muris* rDNA is 61.9%. The G + C content of the LSU-rDNA is 57.2%. An open reading frame of 281 amino acids was found in the spacer DNA.

Structure of the LSU-rRNA

The sequence alignment of the LSU-rDNA including 5.8S rDNA of *G. muris* with that of *G. duodenalis, G. ardeae,* and *E. coli* is presented in Fig. 2. Overall similarity of the LSU-rDNA, not adjusted for secondary structure, was determined from sequence alignment of *G. muris* LSU-rDNA with those of *G. duodenalis, G. ardeae, E. coli,* and *H. halobium* and was 65%, 71%, 46%, and 47%, respectively.

Secondary structure models for *G. muris, G. ardeae,* and *G. duodenalis* LSU-rRNA were constructed. The structure of the *G. muris* rRNA is shown in Fig. 3. Domains (roman numerals) and regions of interest (A–H) are indicated which are based on the secondary structure models described by Gutell et al. (1990). Since the 5.8S domain differs among the three species, the ones for *G. ardeae* and *G. duodenalis* are shown separately in Fig. 4, where the tentative helices A and B are indicated in the *G. duodenalis* structure. The size of the same region in *G. muris* is smaller, 6 nt compared to 19. The same is true for the *G. ardeae* RNA, which has only 5 nt here. The regions where the largest differences were found among the three species, namely C, D, F, and H, are shown separately in Fig. 5.

Region C consists of approximately 34 nt in *G. muris* and 35 in *G. ardeae,* but only 16 nt in *G. duodenalis,* with the helical segment of only 8 nt. Region F shows less difference among the *Giardia* rRNAs, being 20 for *G. muris* and 26 and 28 nt for *G. ardeae* and *G. duodenalis,* respectively. Region H is also larger in these latter two species. Of these three regions, the ones of *G. ardeae* and *G. duodenalis* are more similar to each other than to that of *G. muris.*

Discussion

The entire nucleotide sequence of the rDNA operon of *G. muris* has been determined. The organization of the rDNA of *G. muris* is different from that found in two other *Giardia* species (*G. duodenalis* and *G. ardeae*). The spacer of *G. muris* rDNA is larger than the one in *G. duodenalis* (van Keulen et al. 1991) and it is the only one of the three that is heterogenous in size. The difference resides in sequences upstream of and close to the SSU-rRNA gene. Many tandem repeats are localized in this segment of the rDNA. This phenomenon of tandem repeats in spacer DNA is common in rRNA genes and is believed to be involved in transcription regulation (Jacob 1986; Sollner-Webb and Tower 1986). Why there is heterogeneity in the *G. muris* spacer DNA and not in those of *G. duodenalis* and *G. ardeae* is unclear.

An open reading frame (ORF) in the antisense strand of the *G. duodenalis* LSU-rDNA has been described by Upcroft et al. (1991). There was no evidence for a similar ORF in the *G. muris* sequence. However, an ORF for 281 amino acids was present in the spacer DNA, on the same strand as the rRNA genes, which would yield a protein of about 30 kDa. Whether this ORF codes for a real protein in *G. muris* remains to be determined.

When the SSU-rRNA of *G. duodenalis* was analyzed, the rRNA appeared to resemble in size prokaryotic 16S rRNA. Phylogenetic analysis showed *G. duodenalis* as the earliest-branching eukaryote studied to date (Sogin et al. 1989). A similar observation can be made in the case of *G. muris* and *G. ardeae* (manuscript in preparation). As might be expected, the LSU-rRNA of *Giardia* shows similar features. It is, with respect to its size, similar to prokaryotic rRNA, but contains in its secondary structure many typical eukaryotic features. Notably, these eukaryotic features are among the smallest described so far. Space limitations prevent the discussion of all of the eukaryotic signature features *Giardia* maintains, but a few will be discussed below. For an optimal analysis of similarity and differences among the *Giardia* LSU-rRNA, the secondary structures of these molecules were constructed. The structure of the *G. duodenalis* LSU-rRNA was taken from the sequence determined by

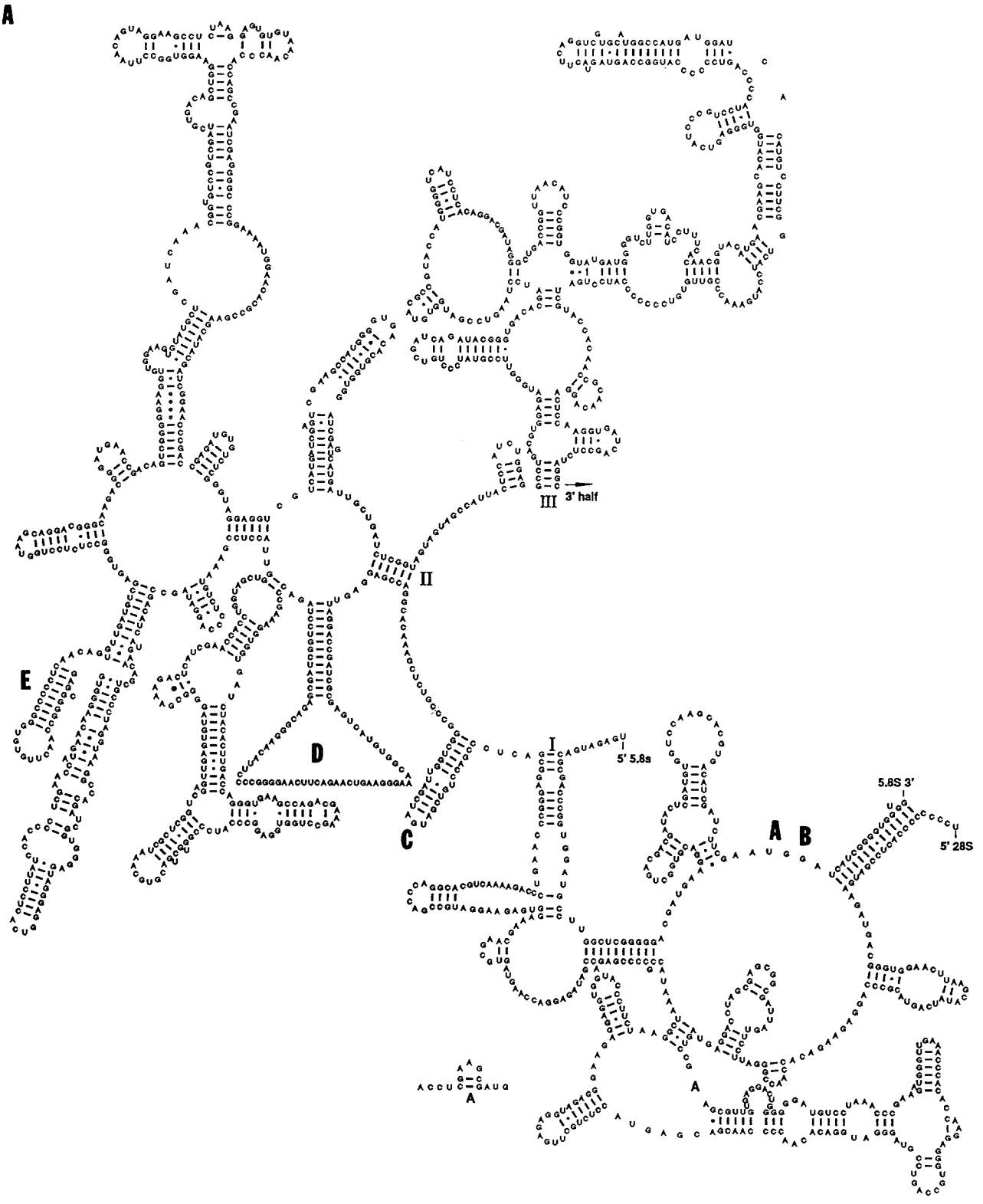


Fig. 3. Secondary structure of the *G. muris* LSU-rRNA. The secondary structure is divided in six domains, indicated with roman numerals (I–VI). The positions of some variable regions are indicated with A–H and are based on the compendium of Gutell et al. (1990). (**A**) Domains I–III and (**B**) IV–VI. Continued on page 324.

Healey et al. (1990), referred to as *G. intestinalis* in their paper. (Minor corrections were made based on our own sequence analysis.) The following general features appeared when the various domains and helices were analyzed.

Domain I

The first feature is what appears to be a 5.8S rRNA that is not a part of the LSU-rRNA as is the case in prokaryotes and *Vairimorpha* (Vossbrinck and

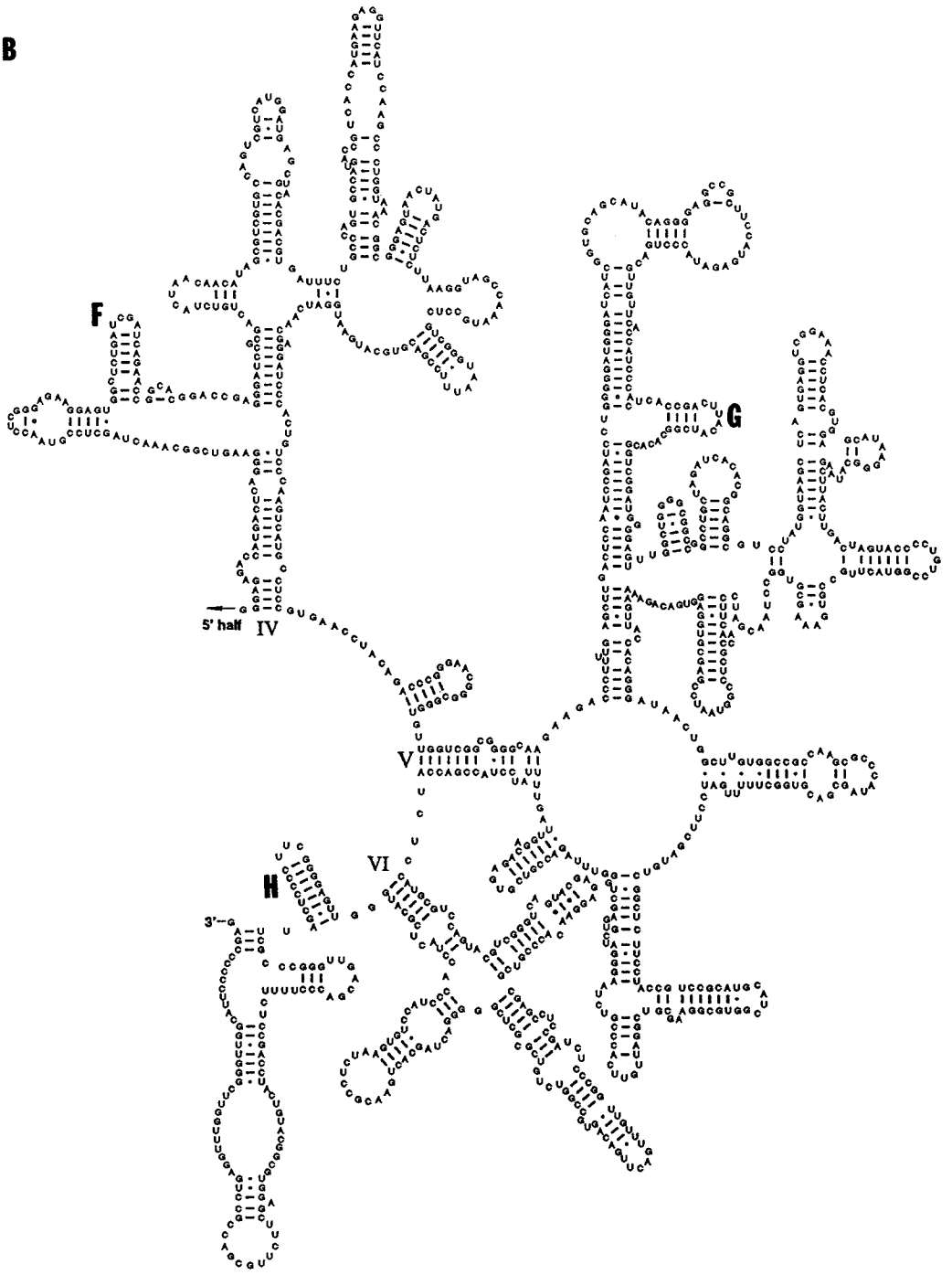


Fig. 3. Continued from page 323.

Woese 1986). It is possible to isolate 5.8S rRNA from *G. duodenalis* (Montanez et al. 1989) and S1 mapping has identified the position of the 5′ end of the LSU-rRNA (Boothroyd et al. 1987). The size of the *Giardia* 5.8S rRNA, however, appears to be smaller than that in other eukaryotes (Edlind and Chakraborty 1987; van Keulen et al. 1991a). Although the exact 5′ and 3′ ends of the RNA are not known, the 5.8S rRNA sequence can be estimated from the base-pairing scheme. A similar, though not identical, structure was obtained by Edlind et al. (1990) for the *G. duodenalis* 5.8S domain. The shorter size of the *Giardia* 5.8S rRNA seems to be the result of shortening of helices A and B in a tentative *G. duodenalis* model. These helices are absent in *G. muris* and *G. ardeae*. An absence of helix A and B is also found in *Pirulla marina;* helix B is absent in many but not all plastid RNAs [see Gutell et al. (1990) for a survey of these irregular helices]. The largest variation in size is seen in region C, also described as domain D2 (Michot and Bachellerie 1987), which is considered a eukaryotic-

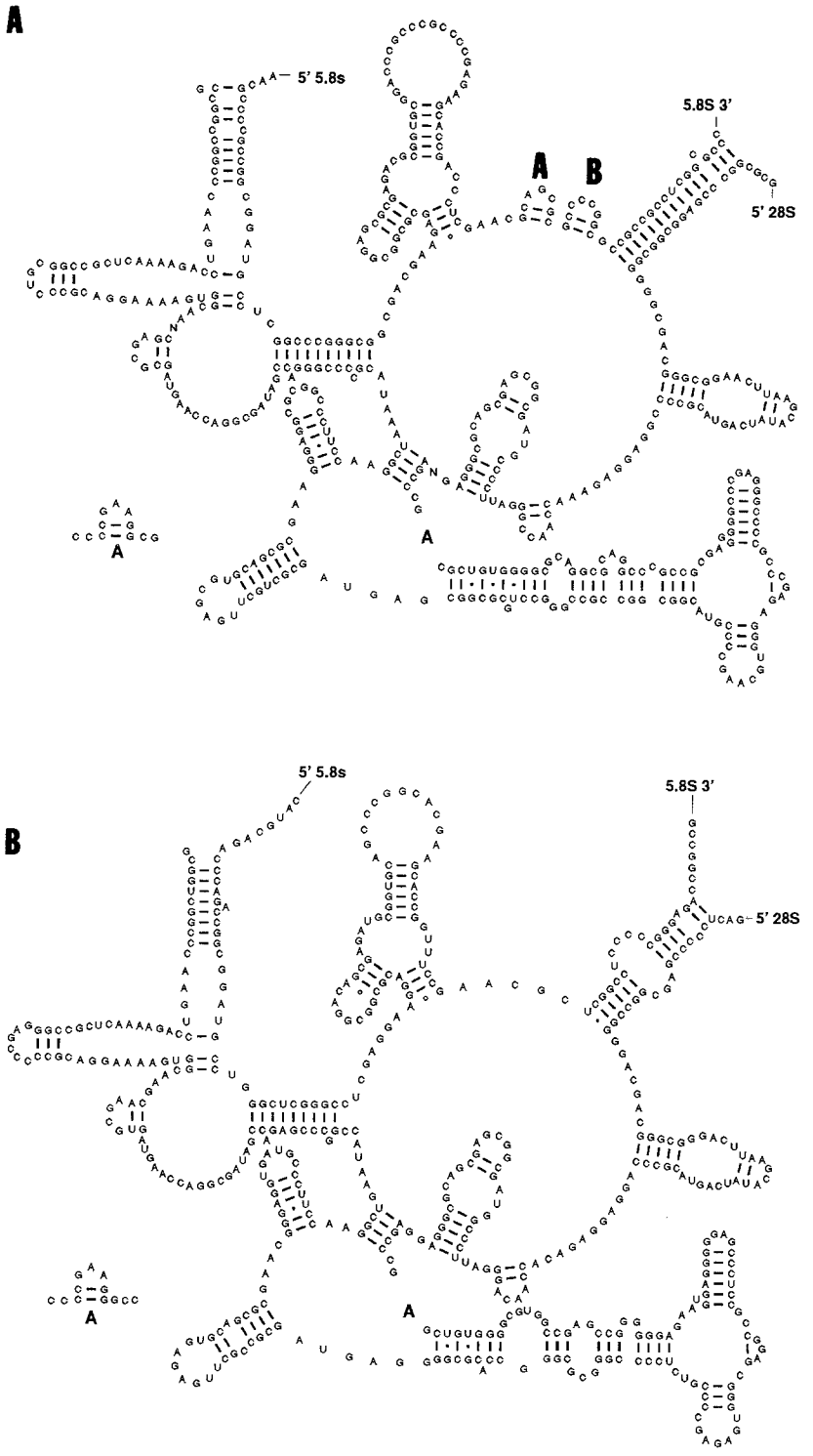


Fig. 4. The structure of the 5.8S rRNA domain. The 5.8S rRNA domain of *G. duodenalis* **(A)** and *G. ardeae* **(B)**. Helices A and B are indicated in the *G. duodenalis* structure.

specific secondary structure element (Michot and Bachellerie 1987). This region in eubacteria is about 30 nt and in archaebacteria about 80 nt. In eukaryotes, however, the size varies from 213 (*Crithidia*)–873 (*Homo*) nt. In contrast to these large sizes, this equivalent region in *G. muris* is only 34 nt and in *G. duodenalis* only 16 nt. The size of this region in *G. ardeae* is almost the same as that in *G. muris* (35 nt). Region C in *G. duodenalis* is the most truncated found so far.

Domain II

Region D is variable in size and secondary structure. This region is similar in *G. duodenalis* and *G. ardeae* in contrast to what is observed for region C. No phylogenetically conserved helix can be formed for D in *G. muris*. Variable region E is generallconsidered kingdom specific and is a typical eukaryotic signature sequence.

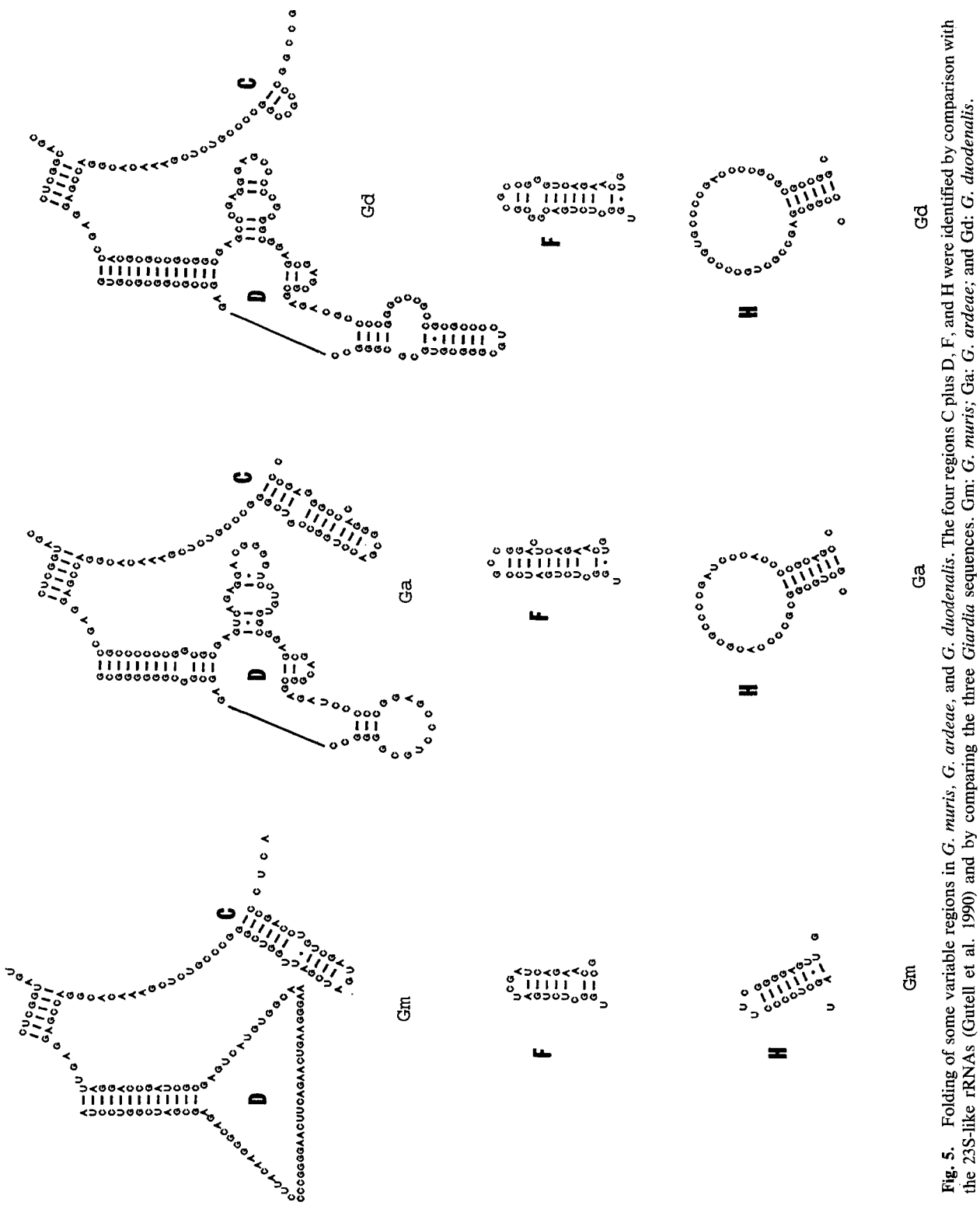


Fig. 5. Folding of some variable regions in *G. muris*, *G. ardeae*, and *G. duodenalis*. The four regions C plus D, F, and H were identified by comparison with the 23S-like rRNAs (Gutell et al. 1990) and by comparing the three *Giardia* sequences. Gm: *G. muris*; Ga: *G. ardeae*; and Gd: *G. duodenalis*.

Domain III

A number of variable regions are present in this domain. In *Giardia,* these appear to be truncated, accounting in part for the small size of the rRNA.

Domain IV

The second characteristically eukaryotic variable region, indicated as F in Fig. 3, or D8 in the nomenclature of Michot and Bachellerie (1987), resides in this domain. It is 45 nt in *E. coli,* 22–37 nt in archaebacteria, and ranges from 143 (*Tetrahymena*) to 718 (*Homo*) nt in eukaryotes, with lower eukaryotes having the smaller size. This region is only 20 nt in *G. muris* and is 28 nt in *G. duodenalis*. In *G. ardeae* it is again similar to that of *G. duodenalis,* namely, 26 nt.

Domain V

Region G is reduced in all three *Giardia* species, with a size (22 nt) which is similar to that of prokaryotes (30 nt in *E. coli*). This region is smaller in *Giardia* than the same region in all other analyzed eukaryotes, where it ranges from 74 (*Tetrahymena*) to 261 (*Crithidia*) nt. The overall structure of this domain and the sequence of the unpaired bases in the central loop are similar to *E. coli* LSU-rRNA, with a high degree of sequence conservation.

Domain VI

This domain shows the least sequence homology to other LSU-rRNA. However, when folded, this domain has, despite being quite variable, a secondary structure that is similar to that same domain in all LSU-rRNA. The putative 3′ end of the LSU-rRNA can therefore be localized with a reasonable degree of certainty. Since it is not possible to isolate enough rRNA from *G. muris* cysts to identify the exact position of the 3′ end of the LSU-rRNA, sequence comparison to *G. ardeae* and *G. duodenalis* was used to estimate the possible 3′ end. The loss of sequence homology between *G. ardeae* and *G. duodenalis* was used as an indication of the 3′ end (van Keulen et al. 1991a). This suggests that the *G. duodenalis* LSU-rRNA ends much earlier than other investigators have reported (Boothroyd et al. 1987; Healey et al. 1990). Based on the putative folding of domain VI, this conclusion still holds. Additional evidence comes from a reevaluation of the size of the LSU-rRNA from *G. duodenalis* by gel electrophoresis of glyoxal-treated RNA which included more markers than previously used. A size of about 1.7 kb was obtained (unpublished results). Based on the alignments of Fig. 2, this agrees well with the observed size of the LSU-rRNA. The major variable region in domain VI is indicated with an H in Fig. 3. This region is much larger in other eukaryotes (64 nt [*Euglena*]–230 nt [*Rattus*] than in *Giardia:* 19 nt in *G. muris* and 38–40 nt in the other two species of *Giardia*.

In conclusion, the size of the LSU-rRNA of *G. muris* is, together with the similar structures of *G. duodenalis* and *G. ardeae* LSU-rRNA, prokaryotic rather than eukaryotic. However, a number of phylogenetic signatures link *Giardia* with eukaryotes. Two variable regions in particular, C and F, but also G and H, are examples of a considerable shortening of the rRNA size where these structures are among the shortest found in eukaryotic LSU-rRNA. All this supports the suggestion that *Giardia* appears to occupy a unique position having one of the most truncated LSU-rRNAs found in eukaryotes so far. This is consistent with other observations to the effect that *Giardia* belongs to the earliest and deepest branching of the eukaryotic evolutionary tree. However, to give *Giardia* the status of "missing link" between pro- and eukaryotes, as others (Kabnick and Peattie 1991) have suggested, cannot be maintained, since *Giardia* LSU-rRNA shows many typical eukaryotic features. The rDNA gene, as a whole, has many distinctly eukaryotic features, being tandemly repeated, having spacer DNA with variable sizes, and containing a separate gene for the 5.8S rRNA. The short size of the entire operon—which is the result of a short spacer, a smaller 5.8S rRNA, and the shortening in size of important structural elements in the rRNAs, as shown here for the LSU-rRNA—and the finding of open reading frames in the rDNA operon, appear to make *Giardia* rRNA genes unique.

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