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Is Higher-Order Structure Conserved in Eukaryotic Ribosomal DNA Intergenic Spacers?

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Summary. Computer-based structural analysis of the ribosomal DNA intergenic spacer (IGS) from the mosquito *Aedes albopictus* revealed a potential to form strong and extensive secondary structures throughout a 4.7-kilobase (kb) region. The predicted stability of secondary structures was particularly high within a 3.15-kb region containing 17 tandem 201 base-pair subrepeats. Similarly strong secondary structure potential was also found when IGS subrepeats were analyzed from 17 phylogenetically diverse eukaryotes, including vertebrates, invertebrates, and plants. Conservation of higher-order structure potential in the IGS region of ribosomal DNA may reflect evolutionary and functional constraints on chromatin organization, transcriptional regulation of the ribosomal RNA genes, and/or transcript processing and stability.

Key words: Ribosomal RNA — Intergenic spacer — Secondary structure — Chromatin organization — Mosquito

Introduction

The tandemly repeated arrays of ribosomal RNA (rRNA) genes in eukaryotes contain two functionally defined regions: the actively transcribed region encoding 18S, 5.8S, and 28S rRNAs, and an intergenic spacer (IGS) region bearing a variety of transcriptional regulatory elements (reviewed in Mandal 1984; Reeder 1989; Sollner-Webb and Mougey 1990). The core domains of the rRNA coding regions have been highly conserved throughout evolution, presumably due to strong selection imposed by structural and functional properties of the ribosome (Gerbi 1985; Gerbi et al. 1987; Clark 1987). In contrast, the expansion segments within eukaryotic rRNA genes have diverged in length and sequence, but nevertheless retain conserved secondary structure (Linares et al. 1991). Finally, the IGS sequences from different eukaryotic rDNA repeats exhibit extensive divergence, which has been correlated with the absence of functional compatibility among RNA polymerases I, associated transcription factors, and the cognate promoters and transcriptional regulatory elements from different species (Grummt et al. 1982; Sollner-Webb and Tower 1986; Ishikawa et al. 1991). Rapid interspecific divergence of RNA polymerase I transcriptional regulatory elements has been attributed to the fact that the enzyme transcribes only the rRNA genes, for which a single promoter type (Sollner-Webb and Tower 1986) undergoes concerted evolution within rRNA multigene families (Dover 1982; Dover and Flavell 1984). Thus, within a species, the evolution of rRNA promoters presumably drives compensatory changes in the associated transcriptional machinery.

Despite their diversity in length and primary sequence, the IGS regions of higher eukaryotes share broadly conserved organizational similarity (Sollner-Webb and Tower 1986; Hemleben et al. 1987; Reeder 1989). Among the most striking of the con-

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Table 1. Conservation of potential secondary structure in IGS subrepeats[a]

Species	bp	kcal/mol[b]	Reference
Aedes albopictus	198	−61.1	Baldridge & Fallon 1992
Drosophila melanogaster	240	−47.9	Simeone et al. 1982
Drosophila virilis	226	−36.4	Murtif & Rae 1985
Drosophila oreana	241	−37.5	Tautz et al. 1987
Drosophila hydeii	226	−42.2	Tautz et al. 1987
Glossina morsitans	420	−79.2	Cross & Dover 1987
Artemia salinella	617	−122.5	Koller et al. 1987
Rat	150	−22.7	Yavachev et al. 1986
Mouse	132	−29.2	Kuehn & Arnheim 1983
Xenopus borealis	138	−52.1	Bach et al. 1981
Xenopus clivii	130	−46.7	Bach et al. 1981
Xenopus laevis	147	−66.2	Moss et al. 1980
Wheat	136	−44.4	Barker et al. 1988
Maize	200	−51.1	McMullen et al. 1986
Rice	266	−105.1	Takaiwa et al. 1990
Tomato	141	−55.7	Perry & Palukaitis 1990
Carrot	465	−141.8	Taira et al. 1988
Radish	124	−24.3	Delcasso-Tremousaygue et al. 1988

[a] For most of the species listed above, multiple subrepeat size classes have been identified. From the arthropod sequences (*Aedes, Drosophila, Glossina,* and *Artemia*), subrepeats containing promoter duplications and/or lying immediately upstream of the true promoter were selected for analysis (subrepeat R16 for *Aedes*). From *Xenopus* species, the contiguous "60/81 bp" subrepeats were analyzed. The largest-identified subrepeat classes were selected from the mammalian and plant sequences. The rat, mouse, and radish subrepeats contained 3′ terminal 14 and 16 nucleotide poly-U and 20 nucleotide poly-A tails, respectively

[b] The minimum free energy values of predicted RNA secondary structures are shown

served structural features are tandem arrays of subrepeating sequence motifs containing "spacer" promoter duplications of a "true" RNA polymerase I promoter, as well as transcriptional enhancers. Typically, the subrepeat lengths correspond to approximately 0.5, 1, 2, or 3 times that of a nucleosomal-core-plus-linker span of DNA. Nuclease protection experiments with *Drosophila* chromatin have revealed a regular 240-bp spacing of nuclease-sensitive sites in the IGS, correlating well with the underlying sequence organization of tandem 239-bp subrepeats (Udvardy et al. 1984). The implication of an "open nucleosome" organization was further supported by studies of cross-linked protein-DNA complexes from *Xenopus laevis,* which showed that the IGS enhancer/promoter region of transcriptionally active rDNA was deficient in histone H1 relative to nontranscribed rDNA (Dimitrov et al. 1990).

A second conserved IGS structural motif is the location of "true" RNA polymerase I promoters downstream of the tandem subrepeat arrays as well as limited sequence similarities within core domains of these promoters (Reeder 1989; Baldridge and Fallon 1992). In some species, it has been shown that this true promoter functions in concert with a nearby transcriptional termination element (McStay and Reeder 1990; Sollner-Webb et al. 1991). The conserved structural organization of homologous transcriptional regulatory elements in IGS regions of higher eukaryotes led us to examine whether IGS sequences from diverse species might share similar secondary structure potential. Computer analysis of the IGS in the mosquito, *Aedes albopictus,* revealed extensive regions of self-complementarity that could generate cruciform DNA structures and/or extensive secondary structure in IGS transcripts. Moreover, comparable self-complementarity found in IGS sequences from other higher eukaryotes may explain the retention of IGS structural organization in spite of phylogenetic divergence in sequence.

Materials and Methods

IGS sequences from an *Aedes albopictus* rDNA repeat (Park and Fallon 1990) were subcloned and sequenced (Baldridge and Fallon 1992) as described previously. The 4.7-kb IGS region was analyzed for secondary structure potential with the MFOLD program (Zuker 1989; Jaeger et al. 1989; Jaeger et al. 1990) using an IRIS 4D/70GT computer. Selected regions from published IGS sequences of other higher eukaryotes were analyzed with the same program as detailed in Table 1. Lack of IGS interspecific sequence identity dictated reliance on the MFOLD program which uses the free-energy minimization technique to predict secondary structure, in preference to the comparative phylogenetic approach which relies on identification of compensatory mutations to confirm structure predictions. Figures were produced with the LoopViewer program by D.G. Gilbert, available via anonymous ftp to iubio.bio.indiana.edu., and minimum free energies are based on RNA structures.

Results

Based on analysis of the 4.7-kb IGS sequence in an *A. albopictus* rDNA clone (Fig. 1), three distinct

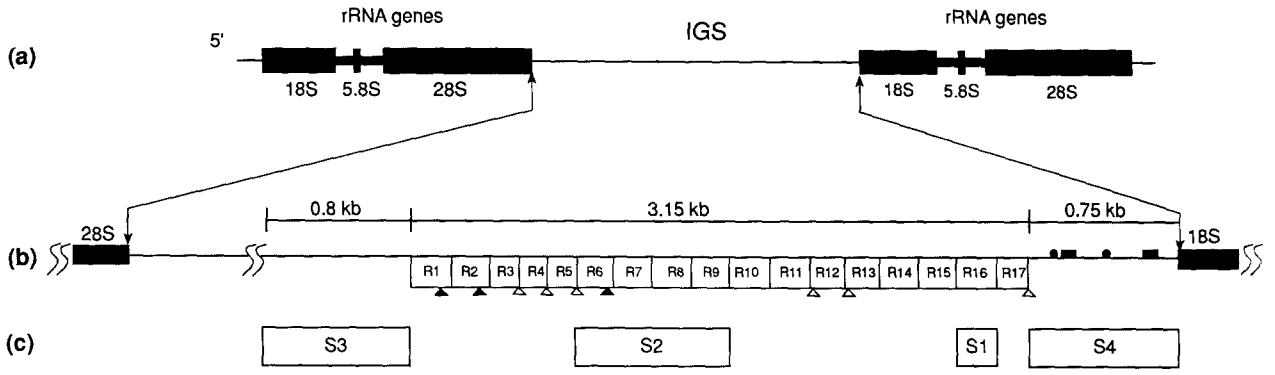

Fig. 1. Structure of a typical eukaryotic rDNA repeat. **a** Two consecutive rRNA coding regions (filled boxes) separated by an IGS region. Note that the gap, which in insects separates the 28S rRNA gene into α and β regions, is not shown. **b** Expanded view of the *A. albopictus* IGS region. The 0.8-kb "upstream" region, the 3.15-kb span of 201-bp subrepeats, and the 0.75-kb transcriptional regulatory region are indicated at top. Within the IGS, the region left of the double-wavy-line symbol has not been sequenced. The seventeen 201-bp subrepeats are represented by boxes below the line. Filled triangles indicate identical 9-bp insertions. Open triangles indicate identical 3′ 54-bp deletions (R3, R4, R5), identical 5′ 21-bp deletions (R12, R13), and a 3′ 84-bp deletion (R17). Filled circles and rectangles above the line indicate putative transcription termination elements and RNA polymerase I promoters, respectively. **c** Relative positions of secondary structures discussed in text and shown in Figs. 2a (S1), 2b (S2), 3a (S3) and 3b (S4).

regions, each with unique characteristics, were defined (Baldridge and Fallon 1992). The 0.8-kb upstream region contained two copies each of three short repeated sequence elements embedded within unique sequence. The central 3.15-kb region contained 17 tandem head-to-tail subrepeats with a consensus length of 201 bp. As described in detail elsewhere (Baldridge and Fallon 1992), identity between the internal subrepeats and the consensus sequence ranged from 80% to 96%, while identity of the 5′ (R1) and 3′ (R17) flanking subrepeats was 60% and 73%, respectively. Among the subrepeats, nonrandom sequence variation defined two families: R1–R8 and R9–R17. With the exception of R17, each subrepeat contained an imperfect complementary (i.e., antisense) duplication of an 11-bp core domain contained within the two putative RNA polymerase I promoters. Finally, within the 0.75-kb downstream region, RNA polymerase I promoters and associated terminator elements were defined on the basis of primary and secondary structural features analogous to those in other species. The occurrence of dyad symmetry throughout the 4.7-kb IGS region, the clustering of 201-bp subrepeats with identical insertions or deletions, and the antisense nature of the putative promoter duplications prompted examination of potential long-range structural interactions in the *A. albopictus* IGS sequence.

Computer analysis predicted extensive secondary structure potential throughout the IGS sequence, particularly in the 3.15 kb containing the tandem array of 201-bp subrepeats. Hydrogen-bonded structures characterized by similar stem-loop patterns were predicted for each of the R9–R17 family of subrepeats (Fig. 2a). The computer-derived minimum free energies (based on RNA structure) ranged from −35.7 kcal/mol for subrepeat R17 to −61.1 kcal/mol for R16 (average: −50.3 kcal/mol). Note that in each of 3 alternative structures predicted for R16 (S1 in Fig. 1c; Fig. 2a), the antisense promoter duplication occurred as part of a stem-loop structure, in which a short T-rich loop occurs as a cap or a bulge. This antisense promoter motif occurred in predicted structures from each of the IGS subrepeats. Moreover, despite minor shifts in the relative positions of stem-loop structures, repeat elements in the R1–R8 family, which contained deletions (R3, 4, 5) or insertions (R1, 2, 6) near the 3′ end of the repeat unit (Baldridge and Fallon 1992), folded into structures that retained overall similarity to those generated by members of the R9–R17 family.

When sequences spanning two to six consecutive subrepeats were analyzed, predicted secondary structures contained stems with up to 37 hydrogen-bonded base pairs as shown for subrepeats R6–R9 (Figs. 1c, 2b). The regular occurrence of long stable stems capped by small loops and joined by three- or four-way junctions was a striking feature of these larger structures in which subrepeats often base-paired with other subrepeats rather than internally against themselves (Fig. 2b). Furthermore, nucleotides at the 5′ end of subrepeats interacted either with the 3′ nucleotides of the previous subrepeat, with adjacent 5′ nucleotides within the same subrepeat, and/or with nucleotides located approximately 50 or 170 bp downstream from the 5′ end of the same or another subrepeat, suggesting that patterns which emerge from primary structure analysis do not necessarily define secondary structure. Minimum free energies of representative structures con-

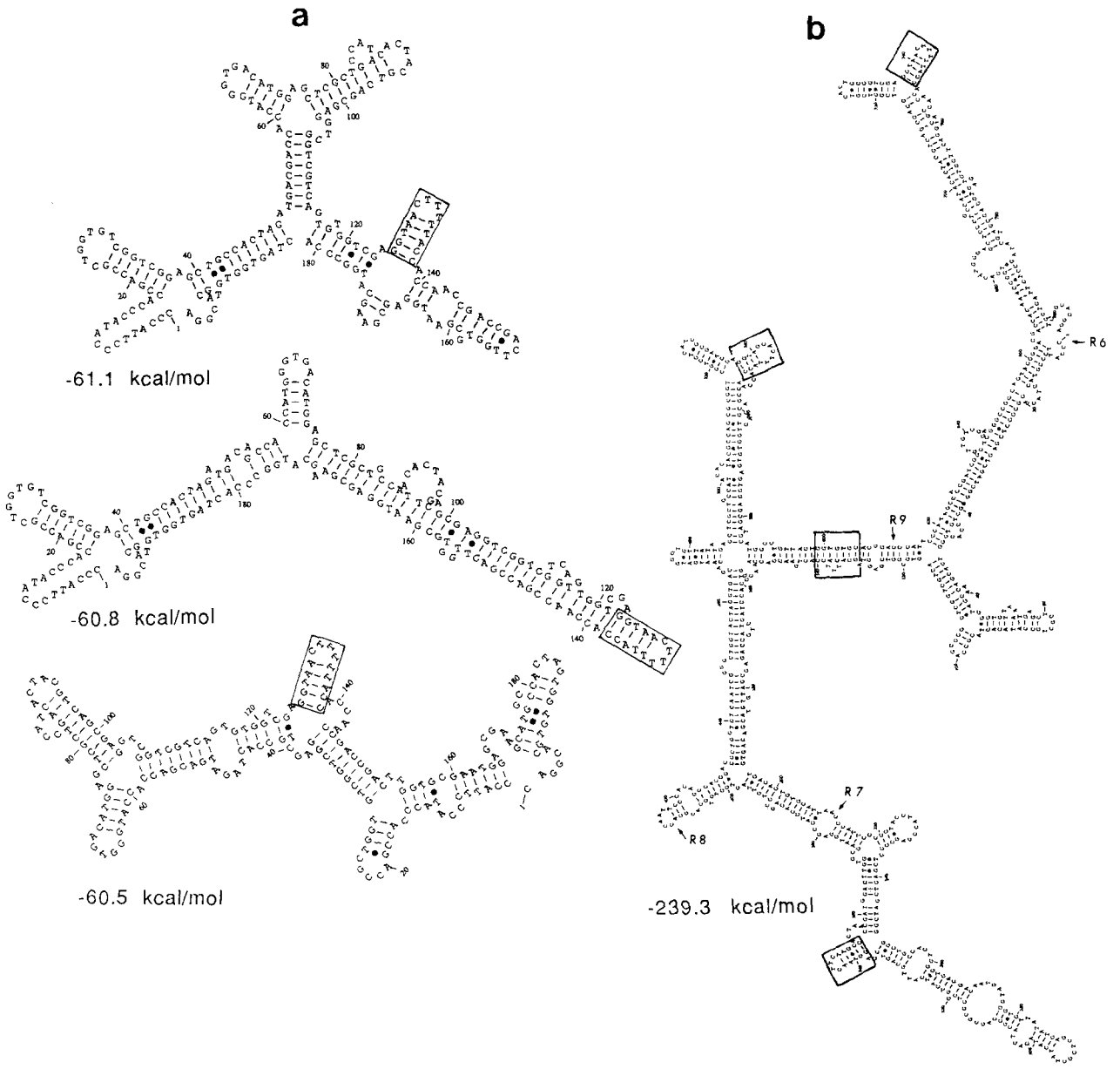

Fig. 2. Representative structures based on *Aedes albopictus* IGS subrepeats. **a** Predicted alternative structures from the R16 sequence (see S1; Fig. 1c). Although DNA structures are shown, free energy values derived by computer are based on predicted RNA structures. Watson-Crick base pairs are indicated by a solid line (e.g., A–T) and noncanonical base pairs by a solid dot (e.g., G · T). Nucleotides are numbered from the 5′ end of each structure, as defined by the organizational pattern shown in Fig. 1. Boxed areas indicate stem-loops that include 11-bp antisense duplications (consensus: AACTTTTTACC) of the putative RNA polymerase I promoter core domains. **b** Predicted structure of the sequence from R6 to R9 (see S2; Fig. 1c). Arrows define subrepeats based on primary sequence.

taining multiple subrepeats ranged from −105.0 kcal/mol for the 417 nucleotides in R1 through R2 to −331.0 kcal/mol for the 1060 nucleotides in R12–R17.

Secondary structure potential in the 803-bp region upstream of subrepeat R1 (S3 in Fig. 1c) was less extensive and of less favorable free energy than that predicted for sequences of similar length (S2 in Fig. 1c) spanning four consecutive 201-bp subrepeats. Within the structure with the minimum free energy (−179.8 kcal/mol) predicted for the 803-bp upstream region (Fig. 3a), 28% of the nucleotides were contained in unpaired loops vs. 19% in the minimum free energy structure (−239.3 kcal/mol) predicted for the 810-bp sequence spanning subrepeats R6–R9 (Fig. 2b).

Extensive secondary structure potential also occurred in the 753-bp region (S4 in Fig. 1c) containing putative transcriptional regulatory elements (Fig. 3b). In the structure with the minimum free energy (−196.1 kcal/mol), the nucleotides corresponding to the putative RNA polymerase I core promoters appeared within stem regions containing unpaired bases and noncanonical base pairs. In con-

trast, the 11-bp sequence with homology to putative antisense spacer promoter duplications (Baldridge and Fallon, 1992) appeared as a short stem-loop structure similar to those in the 201-bp subrepeats (Fig. 2).

Based on visual inspection, similarity among the patterns of stem and loop configurations within secondary structures predicted for the downstream 753-bp regulatory region, the central 3.15-kb region of subrepeats, or the upstream 803-bp region was limited. We thus suggest that there are at least three general domains of secondary structure potential in the *A. albopictus* IGS. Analysis of the first 200 nucleotides of the *A. albopictus* 18S rRNA gene indicated secondary structure in close agreement with the accepted 18S rRNA models, supporting the validity of our IGS structural predictions. The *A. albopictus* IGS sequence does not contain homopurine/pyrimidine tracts that might participate in alternative triplex structures.

To determine whether extensive secondary structure potential was unique to the *A. albopictus* IGS, we analyzed the major IGS subrepeats from 17 phylogenetically diverse species, including invertebrates, vertebrates, and plants. In every case, extensive secondary structures with minimum free energies comparable to those for *A. albopictus* IGS subrepeats were predicted (Table 1 and Fig. 4). The relatively weak minimum free energies for rat, mouse, and radish were partially due to 3′ homopolynucleotide tails that constituted approximately 15% of the length of subrepeats and remained unpaired in the secondary structures (Fig. 4).

Discussion

The conserved potential to form stable secondary structure in IGS sequences may reflect an evolutionary constraint that overrides sequence divergence and general lack of functional compatibility among rDNA transcriptional regulatory elements from diverse organisms. Although the biological role of such structures remains to be explored, the possibility exists that potential of IGS regions to form cruciform DNA structures may underlie a number of apparently unrelated observations. For example, in *Drosophila* polytene chromosomes a single rDNA repeat introduced by P-element transformation organizes a nucleolus and is transcribed, regardless of the site of chromosomal insertion, suggesting that rDNA sequences alone generate nucleolar structure (Karpen et al. 1988). Additional support for a structural role of rDNA derives from the organization of amplified rDNA repeats into extrachromosomal "micronucleoli" that are efficiently transcribed in *Xenopus* oocytes (Miller and Beatty 1969). Electron microscopy studies have further shown that purified rDNA injected into *Xenopus* oocytes generates typical "Christmas tree" structures, in which a nontranscribed region of nucleosome-free DNA precedes the transcribed rRNA genes (Trendelenburg and Gurdon, 1978). These oocyte injection experiments implicated a factor other than RNA polymerase I that allows rRNA genes to be either wholly available or unavailable for transcription, and it seems plausible that rDNA secondary structure may play an intrinsic role in this process. Taken together with these observations from diverse species, the present data support the possibility that extensive secondary structure potential in IGS regions may underlie functional links between nucleolar structure, RNA polymerase I activity, and differential chromatin organization (see the Introduction) in transcriptionally active rDNA cistrons.

At the biochemical level, a functional link between rRNA transcription and DNA secondary structure is supported by the observations that nucleoli are stained by antibodies to cruciform DNA structure (Ward et al. 1991) and are enriched for DNA topoisomerase I (Muller et al. 1985). DNA topoisomerase I has been found to be tightly associated with RNA polymerase I (Rose et al. 1988), and inhibition of topoisomerase I activity using antibodies (Rose et al. 1988) or the drug camptothecin (Zhang et al. 1988) blocks synthesis of the 45S rRNA precursor, suggesting a role for topoisomerase in transcription of rRNA genes (Rose et al. 1988). This functional interaction of topoisomerase and RNA polymerase I supports the potential existence of cruciform structure in IGS rDNA, since cruciform extrusion is influenced by superhelical density (Palacek 1991) which can be altered by active RNA polymerase complexes via topoisomerase-mediated mechanisms (Tsao et al. 1989; Palacek 1991).

Structural organization of the rDNA IGS region may be further influenced by the class of RNA polymerase I transcription factors designated as upstream binding factors (UBFs). The UBFs are important components of the transcription initiation complex at RNA polymerase I promoters, and they also bind upstream spacer promoters and enhancer-bearing tandem subrepeat arrays (Pikaard et al. 1990; Sollner-Webb and Mougey 1990; Sollner-Webb et al. 1991). Based on similarities in amino acid sequence at their carboxy terminal and DNA-binding domains, the UBFs share structural features of nonhistone high-mobility group 1 (HMG1) and 2 proteins (Jantzen et al. 1990; Bachvarov et al. 1991; Hisatake et al. 1991). The HMG1 and 2 proteins have been associated with an "open" nucleo-

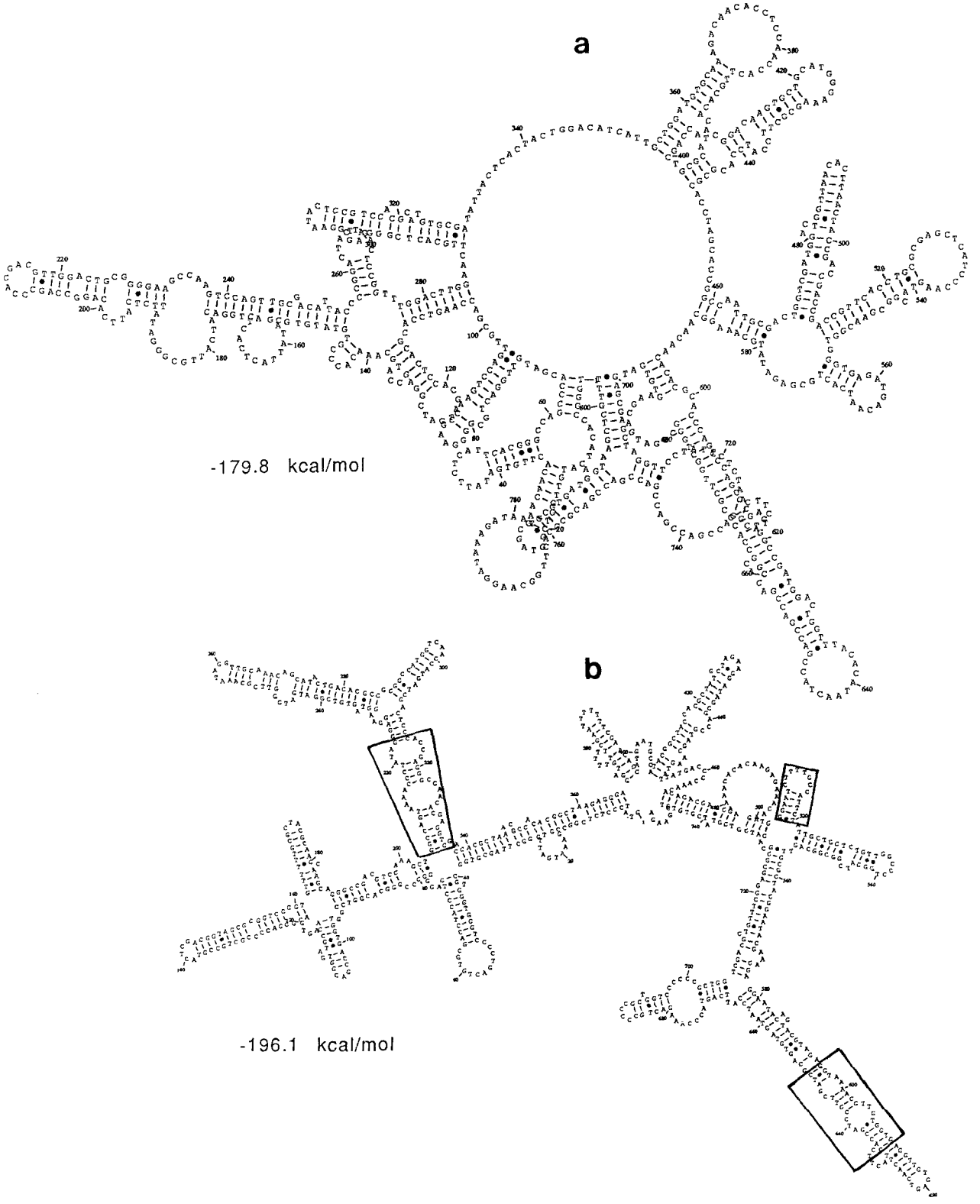

Fig. 3. Predicted structure of *A. albopictus* IGS sequences flanking the tandem array of 201-bp subrepeats. Numerals indicate nucleotide position from the 5′ end of each structure. **a** The 803-bp "upstream" region (see S3; Fig. 1c). **b** The 753-bp region containing putative transcriptional regulatory elements. Core domains of the putative RNA polymerase I promoters as well as an antisense core promoter duplication (positions 505 to 515) are boxed.

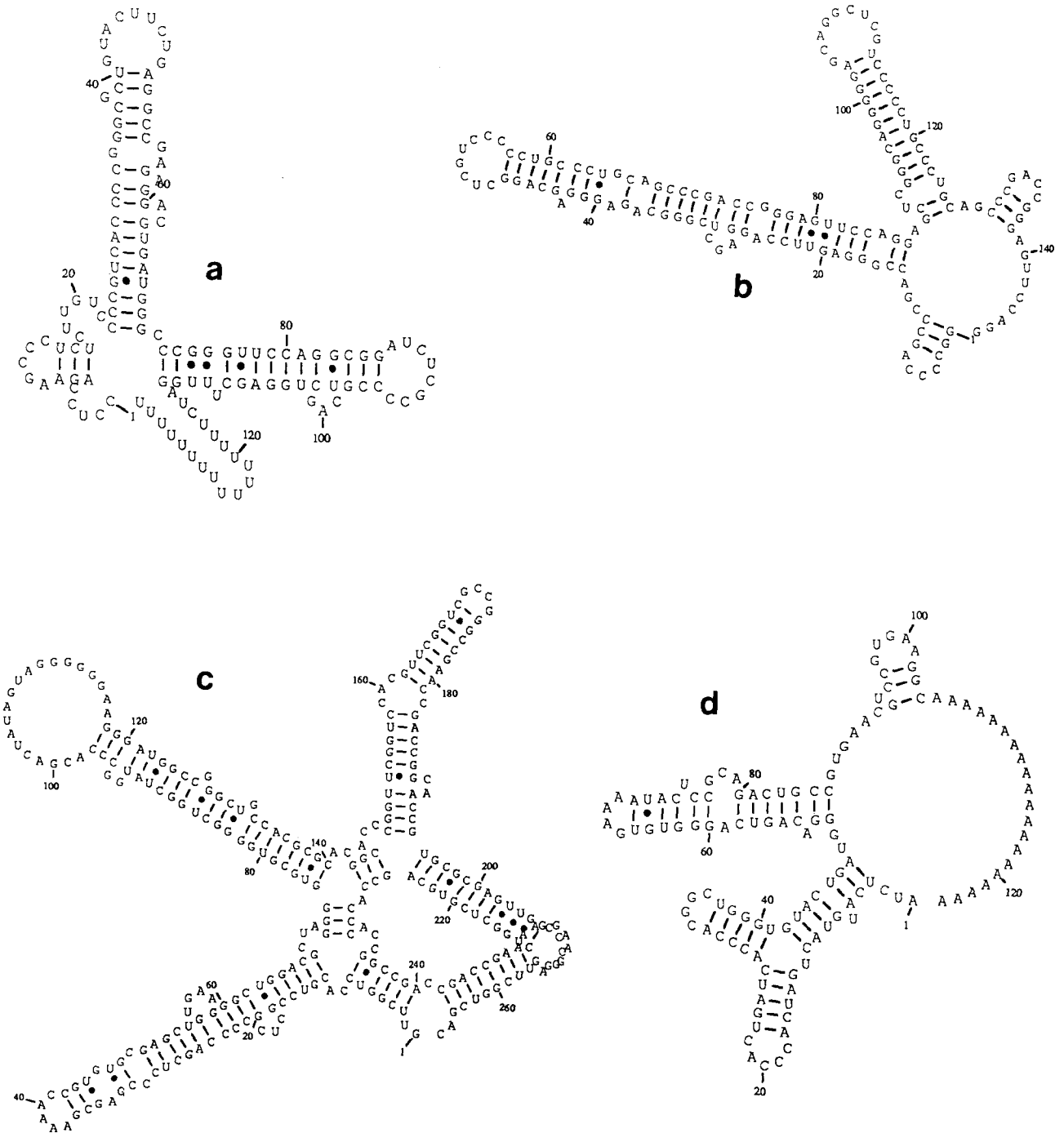

Fig. 4. Predicted secondary structures from single IGS subrepeats (corresponding to *A. albopictus* S1 structures in Figs. 1c and 2a) of representative plant and vertebrate species: **a** mouse, **b** *Xenopus laevis*, **c** rice, **d** radish. Subrepeat lengths, minimum free energies, and references are given in Table 1

some organization and have recently been shown to function as general transcription factors for RNA polymerase II (Singh and Dixon 1990). Similarities between UBFs and HMG1 proteins are of particular interest because human HMG1 has been shown to have a binding preference for single-stranded vs. double-stranded DNA and in addition specifically recognizes and binds cruciform DNA (Bianchi et al. 1989). Moreover, porcine HMG1 has been shown to remove a cruciform-imposed transcriptional block on RNA polymerase II, apparently by binding single-stranded DNA within the cruciform structure and generating a conformational change (Waga et al. 1990). Given their structural similarities, it will thus be of interest to learn whether UBFs share the DNA structural recognition properties of HMG1 proteins and whether the interaction of UBFs with rDNA is influenced by cruciform extrusion. Support for this possibility derives from the recent ob-

servation that UBFs may function as general transcription factors for RNA polymerase I. For example, UBFs from different species bind IGS rDNA in the same approximate positions relative to rRNA promoters, show little sequence specificity, and interact with heterologous rDNA to form functional initiation complexes with heterologous RNA polymerase I and other transcription factors (Pikaard et al. 1990; Bell et al. 1990). Thus, it is intriguing to speculate that UBFs and possibly other transcription factors may interact with IGS secondary structure to regulate rRNA gene expression. The recent demonstration that the active component of an RNA polymerase III transcription factor from the silkworm, *Bombyx mori,* is composed of RNA (Young et al. 1991) underscores the need to consider novel mechanisms of transcriptional regulation.

An alternative interpretation of the biological significance of secondary structure in IGS regions invokes the rDNA "spacer" promoters, which typically occur near or directly within IGS subrepeats and range from highly truncated to almost exact copies of the true promoter (Sollner-Webb and Tower 1986; Reeder 1989). Although difficult to quantify due to their short half-lives, transcripts that initiate at spacer promoters can be detected in vitro or in transfected cells. Our results are compatible with the possibility that transcripts from spacer promoters assume secondary structures that influence their stability. Recent observations have shown that in higher eukaryotes RNA secondary structure influences mRNA stability (Mullner and Kuhn 1988; Stern et al. 1991) as well as tissue-specific mRNA splicing (Clouet D'Orval et al. 1991; Libri et al. 1991). Furthermore, the minimum free energies of potential secondary structures from IGS subrepeats (Table 1) exceed those in the range of -5 to -20 kcal/mol, which are known to influence various aspects of transcription, translation, and mRNA degradation in prokaryotes (Kubo et al. 1990).

Recent computer-based analysis of eukaryotic 28S rRNA expansion segments has revealed conservation of secondary structure within vertebrates (Hancock and Dover 1990) and within higher Diptera (Linares et al. 1991). Evolutionary mechanisms proposed to account for the conserved secondary structure included compensatory base mutations and a novel process termed compensatory slippage in which DNA polymerase presumably generates repetitive nucleotide motifs. If complementary repetitive motifs are capable of forming stable stems that do not disrupt preexisting secondary structure, then they are retained through natural selection (Hancock and Dover 1990). However, the *A. albopictus* IGS does not appear to contain repetitive complementary motifs similar to those described for vertebrate rRNA expansion segments (Hancock and Dover 1990), nor was there substantial evidence for compensatory base mutations within the IGS subrepeats. Most sequence variation among subrepeats occurred in unstable stem structures, bulges, or loops. Furthermore, the IGS differs from expansion segments in that it is not defined and constrained by contiguous coding regions, and, among species, exhibits considerably more sequence divergence. With the available information, it is therefore difficult to propose a mechanistic basis for conservation of IGS secondary structure. Analysis of mosquito rDNA cistrons from *Aedes aegypti* (Gale and Crampton 1989) and various members of the genus *Anopheles* (Beach et al. 1989; McLain and Collins 1989) is underway, and we anticipate that structural comparisons among these genera may enrich the analysis presented here, possibly facilitating cladistic comparisons.

In contrast to the results presented here, most previous observations of secondary structure potential in IGS rDNA, particularly in plants, have been confined to relatively short regions and have focussed on a possible role in transcriptional termination. However, the striking conservation among phylogenetically diverse species of potential to form strong secondary structure extending over several hundred base pairs in IGS regions of rDNA and/or in RNA transcripts suggests a broader role independent of primary sequence. As more sophisticated approaches become available to probe chromatin structure, analysis of rDNA secondary structure may contribute to an overall understanding of nucleolar organization, to regulation of rRNA gene expression, and to the underlying mechanisms that have guided the evolution of these processes.

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