Mouse rDNA: Sequences and Evolutionary Analysis of Spacer and Mature RNA Regions

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Two regions of mouse rDNA were sequenced. One contained the last 323 nucleotides of the external transcribed spacer and the first 595 nucleotides of 18S rRNA; the other spanned the entire internal transcribed spacer and included the 3' end of 18S rRNA, 5.8S rRNA, and the 5' end of 28S rRNA. The mature rRNA sequences are very highly conserved from yeast to mouse (unit evolutionary period, the time required for a 1% divergence of sequence, was 30×10^6 to $100 \times$ 10° years). In 18S rRNA, at least some of the evolutionary expansion and increase in G+C content is due to a progressive accretion of discrete G+C-rich insertions. Spacer sequence comparisons between mouse and rat rRNA reveal much more extensive and frequent insertions and substitutions of G+C-rich segments. As a result, spacers conserve overall G+C richness but not sequence (UEP, 0.3×10^6 years) or specific base-paired stems. Although no stems analogous to those bracketing 16S and 23S rRNA in Escherichia coli pre-rRNA are evident, certain features of the spacer regions flanking eucaryotic mature rRNAs are conserved and could be involved in rRNA processing or ribosome formation. These conserved regions include some short homologous sequence patterns and closely spaced direct repeats.

rDNA is evolutionarily conserved as a multigene family. The long precursor rRNA transcripts always contain a spacer region at the 5' end (external transcribed spacer) followed by the rRNA sequence for the smaller ribosomal subunit (16S to 18S). Another spacer (internal transcribed spacer [ITS]) is then transcribed before the rRNA for the larger subunit (23S to 28S). In addition, smaller RNAs usually interrupt the internal spacer: specific tRNAs between the bacterial 16S and 23S rRNAs, and 5.8S rRNA between the eucaryotic 18S and 28S rRNAs. The precursor RNA is cleaved at a number of sites to yield the mature RNAs, and spacer sequences are totally degraded during processing (16).

As one might expect for a structural RNA, the similarity in precursor organization and processing extends to features of the mature rRNA primary sequence. For example, particular bases are methylated, and some sequences are highly conserved in similar rRNAs from different species (8). Conservation of secondary

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structure features has also been suggested (9). The high degree of evolutionary relatedness among mature rRNAs is undoubtedly due to their important role in the synthesis of proteins.

Nevertheless, mature rRNA species tend to become longer and, among the eucaryotes, increasingly G+C rich during evolution, for unknown reasons. For example, the *Escherichia coli* 23S rRNA is 3,000 nucleotides long with 53.5% G+C; the human 28S rRNA is 4,200 nucleotides long with 68% G+C (8). Transcribed spacer sequences show an even greater increase in size and G+C content during evolution. The number of spacer nucleotides in the primary rRNA transcript increases from about 1,400 in *E. coli* to about 6,800 in humans, and the G+C content increases 1.5-fold (8).

Unlike mature rRNAs, however, transcribed spacer sequences diverge rapidly. Not only is there no detectable homology between yeast and *Xenopus laevis* spacers, but eucaryotic spacers have no known function. In contrast, *E. coli* spacer sequences flanking 16S and 23S rRNA base pair to form double-stranded stems which are the sites of initial cleavages in *E. coli* prerRNA (4). Such stems have not been detected in either yeast or *X. laevis* pre-rRNAs, but eucaryotic spacer sequences may have other conserved features that might specify processing cleavage sites, regulate the structure and replication of rDNA, or control transport of nascent ribosomes to the cytoplasm.

To identify conserved features and the basis of the evolutionary trends in rDNA length and G+C content, we have sequenced 3,282 nucleotides of mouse rDNA; the regions include the entire internal transcribed spacer and the sequence surrounding the 5' terminus of 18S rRNA (see Fig. 1). The primary and possible secondary structures of the mouse sequences were then extensively analyzed and compared with those from *E. coli*, yeast, *X. laevis*, and rat.

These analyses suggest that the primary sequence of the ITS diverges very rapidly. However, high G+C content and a capacity to form stable secondary structures are maintained in spacer sequences by the continued insertion and deletion of short G+C-rich regions. Other structural features are evident which, combined with the analysis of pre-rRNA processing sites, permit a search for possible cleavage signals (2, 3).

MATERIALS AND METHODS

Mouse rDNA sequencing. Regions near the termini of mouse (MOPC myeloma) 18S, 5.8S, and 28S rRNAs were sequenced from rDNA subclones 5A, 6, and 7, all containing inserts into plasmid pBR322 as described by Bowman et al. (2). Each subclone was mapped extensively with restriction enzymes; those sites used for end labeling of sequenced fragments are indicated in Fig. 1. Sequencing was essentially as described by Maxam and Gilbert (19), with restriction fragments 5' end labeled with polynucleotide kinase. Labeled fragments were extracted from polyacrylamide gels by electroelution (B. K. Saha, S. Stretlow, and D. Schlessinger, J. Biochem. Biophys. Methods, in press) and were suitable for subsequent restriction enzyme cleavage, strand separation, or sequencing. Computer-predicted restriction sites calculated from the final confirmed sequence matched precisely with actual cleavage patterns for 31 restriction enzymes.

Computer analysis. Sequence editing and restriction enzyme analysis were facilitated by modifications by W. Barnes (Washington University School of Medicine) of the programs of Staden (26). Dot matrix analysis of sequence homologies (see Fig. 5) was generated by a program of W. Barnes and G. Zyda (unpublished, Washington University School of Medicine). More specific alignment of the rDNA sequences was accomplished with the aid of a gapping program (15; adapted for use here by M. Brandenberg) which selects regions of high homology and allows the user to introduce deletions for the best fit of those regions. Like the dot matrix program, this program was most useful for the rDNA sequence when we searched for regions with homology of at least 20 out of 30 bases. Prediction of possible pre-rRNA secondary structure was assisted by the SEQ program of the MOLGEN project (Stanford University) at the National Institutes of Health SUMEX-AIM facility. Dyad symmetries constructed by SEQ were plotted on a matrix (see Fig.



sequenced. Arrows represent length and direction (5' to 3') of sequence derived from these fragments

7) with an adjunct program by M. Brandenberg. This program sorts the data into groups based on the predicted total free energy of each dyad symmetry and then plots the locations of base-paired regions with respect to each sequence. Additional programs by M. Brandenberg aided in displaying homologous sequence alignment and permitted searches for perfect and imperfect sequence repeats. The SEQ program was also used to determine nucleotide frequencies and to help identify homologous sequences between species.

Calculations of the rate of rDNA evolution. Sequence alignments of mature rDNA from different species and ITSs from mouse and rat have permitted us to estimate the rate of evolution for these regions. In calculating conservation and divergence of a given pair of sequences, a gap introduced in the alignment was scored as a single mismatch, and the percentage of divergence was corrected for multiple base change events (20). From fossil records of evolutionary divergence of species (10, 18), the corrected percentage of divergence and bused to estimate the unit evolutionary period (UEP), the time (in millions of years) required to accumulate a 1% divergence between two initially identical sequences (36).

RESULTS

Mouse rDNA sequences. Figure 1 shows the strategy used for sequencing the indicated rDNA segments. The Maxam-Gilbert method was adequate for these studies, although the high G+C content of the internal spacer tended to produce compressed bands on gels. Therefore, repeated analyses of both strands from multiple restriction sites was often necessary to yield an unequivocal sequence. The sequences derived are listed in Fig. 2. Termini of the mature rRNAs were located along the rDNA sequence from their previously determined terminal sequences (13, 14) or by S1 nuclease mapping techniques (2, 3). The G+C content of the various mature rRNA segments varied from 45.9 to 56.2%; the G+C content of spacer segments was much higher, ranging from 70.1 to 75.3%.

Analysis of mature rRNA sequences. The sequences determined for portions of mouse 18S, 28S, and 5.8S rRNAs were compared with the published sequences of *E. coli* (5), yeast (21, 22, 25, 33, 34), *X. laevis* (17, 23, 24), and rat (7, 29). As expected, alignment of these sequences (Fig. 3) indicates that the segments of mouse mature rRNAs are highly homologous to the mature rRNAs from other eucaryotic species (70 to 87% homologous to yeast, 89 to 100% homologous to *X. laevis*). This suggests that the majority of these sequences are critical for eucaryotic ribosomal assembly, structure, or function. In contrast, mouse and *E. coli* rRNAs were only 45.4%homologous in the best computer-assisted alignment of this region, although some regions of better homology were evident, such as nucleotides 367 through 384 and 767 through 820.

Of particular interest are the very G+C-rich insertions in mouse 18S rRNA as compared with X. laevis or yeast 18S rRNA. Figure 4 shows that the size and number of these insertions progressively increase from yeast to mouse sequences and that these insertions can form stable hairpin structures. The increasing size of mature rRNA during evolution may thus be due, at least in part, to the insertion of these G+Crich sequences (see below).

Analysis of spacer rRNA sequences. The transcribed spacer sequences have diverged so rapidly that no alignment could be constructed between mouse and X. laevis or between mouse and yeast ITS sequences. Only when the mouse ITSs were compared with those of rat could an alignment be generated. Figure 5 displays the mouse-rat ITS comparison on a matrix, in which every line indicates a sequence homology of at least 20 out of 30 consecutive nucleotides. Because the ITS sequences are of comparable length in mouse and rat, homologies near the diagonal of the graph are regions similar in location as well as sequence.

An optimized sequence comparison of the mouse and rat ITS regions was derived from Fig. 5 and from searches with a computer program that introduces gaps to improve sequence alignment. The result (Fig. 6) indicates which stretches of sequence are more conserved between the two species; a histogram below the matrix plot in Fig. 5 shows the extent of divergence across segments of the spacer (calculated from the alignment of Fig. 6). The histogram also maps four regions (a, b, c, and d) of the rat ITS previously identified as highly homologous (~75%) to X. laevis ITS sequences (29). However, none of these regions corresponds to those ITS sequences that are the most conserved between rat and mouse. We have not found any long spacer sequences that are highly conserved in all three species.

The spacer sequences near the termini of mature mouse and rat rRNA sequences are no more homologous than other regions of their ITSs. However, a role for these particular sequences in rRNA processing or ribosome formation is more likely because of homologies with X. laevis flanking spacer sequences; the similarities extend for 10 to 20 nucleotides after the 3'

FIG. 2. Mouse rDNA sequence. Termini of mature RNA determined from known rRNA sequences (see text) and from S1 nuclease analysis (2). ETS, External transcribed spacer. Note that the sequence of 18S rRNA is interrupted at the vertical line (nucleotide 918); the remaining 18S sequence is from the 3' end of the rRNA.

18 28	38 4	8 58	60	78	BB	90 100	
9TC6AC0TTCC60CTCTCCC0AT6	CCGAGGGGTTCGGG4	111818CC8886AC8	GAGGGGAGAGC	6661 AASAGAGO	STGTCGGAGAG	CTGTCCC66666C6	
110 120	130 14	IG 150	1 60	178	188	199 200	ETS
ACGCTCGGGTTGGCTTTGCCGCGT	86676766766666	ICGGGTTTTGTCGGAC	:CCCG AC60 GGT	CGGTCCGGCC66	CATOCACTOTO	CCGTTCCGCGCGG	
ZIO ZZO	230 24	0 250	260	278	200	290 300	
COCCOCCCOOCTCACCCCCOOTTT	STCCTCCCGCGAGGC	TCTCCACCOCCOCCO	CCTCCTCCTCT	CTC6C6CTCTC1	T&TTCCGCCT#	GTCCTGTCCCACC	
916 320	330 34	IO 350	368	378	300	390 400	
TT 20 20 2 7 20 T 20 20 20 20 20 20 20 20 20 20 20 20 20	ACCT0GTTGATCCT0	ICCAGTAGCATATGCT	TGTCTCAAAGA	TTAAGCCATGC	ATGTCTAAGTA	CGCACGGCCGGTA	
410 420 CASTGAAACTGCGAATGGCTCATT	4 30 44 MAATCAGTTATGGT1	0 450 CCTTTGGTCGCTCGC	460	478 TTGGATAACTG	400 1661AATTCT#	450 500 GAGCTAATACATG	
518 528 CCGACGOGCGCTGACCCCCCTTCC	538 54 CGGGGGGGGGATGCG1	GCATTTATCAGATCA	568 MAACCAACCCG	578 GTGAGCTCCCT	588 CCCGGCTCCGG	590 600 CCGGGGGGTCGGGC	
610 628 GCCGGCGGCTTTGGTGACTCTAGA	630 64 TAACCTCGGGCCGAT	GCACOCCCCCGTG	668 668666666666	678 CCCATCGAACG	690 ICTGCCCTATC	690 700 AACTITICGATGET	18 S
718 728	730 74	NO 750	7 68	778	788	790 800	
GGTCGCCGTGCCTACCATGGTGAC	CACGGGTGACGGGG	NATCAGGGTTCGATTC	:C GGAGAGGG AG	ICCTGAGAAACG	BCTACCACATO	CAAGGAAGGCAGC	
818 828	030 04	0 030	968	878	BBB	898 988	
AGGCGCGCAAATTACCCACTCCCG	ACCCG666A6GTA61	GACGAAAAA TAACAA	TACASSACTCT	TTCGAGGCCCT	STAATTGGAAT	GAGTCCACTTTAA	
918	930 94 GAAGACOGTCGAACI	IS 958 ITGACTATCTAGAGGA	968 INGTAAAAGTCG	978 TAACAAGGTTT	988 CCGTAGGTGAA	998 1888 CCTGCGGAAGGAT	
1818 1828	1830 184	10 1050	1 868	1878 1	1000 1	898 1188	
CATTACCOCONSACTOTOGASEAS	C66C66C6T66CCC6	ICTCTCCCCGTCTTGT	GTGTGTCCTCG	CCG66A66C6C6	5T6CGTCCCGG	GTCCCGTCGCCCG	
1118 1128	1130 114	10 1150	1160	1170 1	100 1	190 1200	
CGTQTQQQQCGAGGTGTCTQGQQT	GAGGTGAGAGAAGGG	167666766676667	CT666TCC6TC	TGGGACCGCCTC	CGATTTCCCC	TCCCCCTCCCCTC	
1210 1220	1230 124	18 1258	1268	1270 1	1200 1	290 1300	
TCCCTCGTCCGGCTCTGACCTCGC	CACCCTACCGC66C	19666667667666666	CGTCTTGCCTC	TTTCCCGTCCGG	SCTCTTCCGT0	TCTACGA05G6C6	
1310 1320	1330 134	10 1350	1368	1370 1	1380 1	398 1488	
GTACGTCGTTACGGGTTTTTGACC	CGTCCCGGGGGGCGT1	COGTCGTCGGGGGCGC	GCGCTTTGCTC	TCCCGGCACCC	NTCCCCGCC90	GGCTCTGGCTTTT	
1410 1420 CTACGTTG6CT66G6CC00TT6TC6	14 30 144 CGT GTGGGGGGGATG T	B 1458 GASTETCGCETETGG	1468 GCTCGCCCGTC	1478 S	14 88 1	498 1588 TC9C6T6TCCTCC	
1510 1520	1530 154	ID 1550	1568	1578 1	1588 1	598 1688	1151
CCGCTCCTGTCCCGGGTACCTAGC	TGTCGCGTTCCGGCG	ICOGAGGTTTAAGACC	CC66666TC6C	CCT6CC6CCCC	CAGOGTCGGGG	GGCGGTGGGGCCC	
1610 1620	1630 164	B 1650	1660	1670 1	1 600 1	698 1788	
GTAGGGAASTC889C6TTC688C8	BCTCTCCCTCABACT	CCATGACCCTCCTCC	CCCCGCTGCCG	CC6TTCCCGAGG	16 00 CGGTCGT	GT6606666T66A	
1718 1728	1730 174	0 1750	1760	1778 1	1786 1	798 1888	
TGTCTG909CCCCCTCTG69CGCC	CGTGGGGGGCCCGAC	COCGCCGCCGGCTGC	CGATTTCCGCG	GGTCGGTCCTG1	ICGGTGCCGGT	CGTG6GTTCCCGT	
1818 1828	1830 184	0 1850	1 868	1676 1	1	990 1980	
STCSTTCCCGTSTTTTTCCGCTCC	CGACCCTTTTTTTT	CCTCCCCCCCACACE	16TCTC6TTTC	STTCCTTSCTG	3CCT66CCT64	GGCTACCCCTC66	
1910 1920 TCCATCTOTTCTCCTCTCTCCG	1930 194 GOGAGAGGAGGGGGG	1958 11067C077666666AC	1 968 :TGTGCCGTCGT	1970 S	I 900 1 NGTTCGCTCAC	998 2888 ACCCGAAATACCG	
2010 2020 ATACGACTCTTAGCGGTGGATCAC	2030 20 TC66CTC6T6C6TC	10 2050 IATGAAGAACGCAGCT	2868 NOCTOCEMBAA	2070 2	2000 2 TSCAGGACACA	TTEATCATCEACA	500
2110 2120 CTTCGAACGCACTTGCOGCCCCOG	2130 214 6TTCCTCCC0000C	IO 2150 INCOCCTOTCTONGCO	2160	2178 a	2100 2 NCCCGCTGCGG	198 2288 TGGGTGCTGCGCG	5.85
2210 2228 SCT68GAGTTT6CTC6CAGG6CCA	2230 22	10 2250 STC666CCCTCC6TCT	2268 CCCGAAGTTCA	2278 2 GACGTGTGGGCC	2288 2 36TTGTCGGTG	298 2388 TGGCGCGCGCGCC	
2310 2320 CGCGCGCGAGCCTGGTCTCCCCCG	2330 23 COGCACCCGCGCCG	10 2350 1990110110006010	2368 :CGCCCGTTCCC	2370 a	2 380 2 Igcgccccggt	398 2488 CCT60CCTC6C6T	
2410 2420 CGGCGCCTCCCGGACCGCTGCCTC	2430 244 ACCAGTCTTTCTCG	18 2458 BTCCC6T6CCCC6T64	2468 IGAACCCACCGG	2478 a	24 90 2 BCCCGGGGGGTG	498 2588 BGCGCGTCCGCAT	
2510 2520 CTGCTCT00TCGAGGTTGGC0GTT	2530 254 GAGGGTGTGCGTGCG	19 2558 ICCGAGGTGGTGGTCG	2568 IGTCC0C6CT6C	2578 a	25 88 2 TGTCGGGGTGG	590 2600 CGGTCGACCGCTC	
2610 2620	2630 26	18 2638	2668	2670 i	26 00 2	698 2788	1152
0C0666TT00C6C60TC6CCC06C	GCCGCGCACCTCC	BGCTTGTGTGGAGGGA	NGAGCGAGGGCG	ingnacggagagi	NGGTGGTATCC	CC66T66C6T78C	
2718 2728	2730 274	18 2758	2768	2776 2	27 88 2	798 2888	
GAGGGAGGOTTTGGCGTCCCGCGT	CCGTCCGTCCCTCC	TCCCTCGGT6GGCGC	CTTC0C0C0C0C	CGCGGCCGCTA	9660CGGTCG6	886CCCGT86CCCC	
2010 2020 C6T66CTCTTCTTCGTCTCC6CTT	2030 20 CTCCTTCACCC000	18 2858 EGGTACCCGCTCCGGC	2060 :0cc00ccc6c0	2070 a		898 2988 CGATGCGASTCAC	
2918 2928 CCCC00010110C0A011C0000A	2938 29 GGGAGAGGGCCTCO	18 2958 CTGACCCGTTGCGTCC	2 968 :C 0 6CTTCCCT0	2976 (2 988 2 9666TCTGT96	998 3888 GCTGTGC6TCCC6	
3818 3828	3030 30	18 3858	3050	3070 ::	2000 3	050 3100	
6096TT6C6T6T6A6TAA6ATCCT	CCACCCCC6CC6CC	TCCCCTCCC8CC860	CTCTC06666AC	:CCCCT6A6ACG	0TTC6CC06C1	C6TCCTCCC6T6C	
3110 3120 CGCCGGGTGCCGTCTCTTTCCCGC	3138 31 CCGCCTCCTCGCTC	10 3158 TCTTCTTCCCGCGGC1	3160 1606c6c67670	3170 :: ::::::::::::::::::::::::::::::::::		198 3288 AGATCAGACGTGG	200
3218 3228 CBACCCOCTBAATTTAABCATATT	3238 32 ASTCASCOGAGGAA	18 3258 MAGAAACTAACCAGGA	3260	3278 1	3200 ACAGEG		203

	ETS+I8S	358
Mouse Rat	CCCGACGCTT-GCTCGCGCCTTCCTTACCTGGTTGAT	CCTGCCAGTAG-CATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTACGCACG
Xenopus Yeast E.coli	G GCCGGGCC-CGGGAAAGG GGC GTT CTT -CT TTAAGA AG T ATTCTTTGA- GTCAAGA TT AAATTC AGA T	C G TAAG A
		A STCHER-TTERACEC & GEC & CC CA A &
	400	
Nouse Xenopus	GCCGGTACAGTGAAACTGCGAATGGCTCATTAAATCAGTTA	ITGGTTCCTTTGGTCGCTCGCTCCCTCCTACTTGG-ATAACTGTGGTAATTCTAGAGCT
Yeast E.coli	ATTTA G TA CA AAG AGCTTG TTC 1	C TA AAT CT TAC A T C C ga gaagt g ga ggtgag aa gtc -ga cctg tggagg g a
	5.86	
Nouse	ANTACATOCCORCOGOCOCTOACCCCCTTCCCCGOGOGOGO	550
Xenopus Yeast	AA TTAAAAT TT	C T G CC C
E.coli	CTAC GAA TA A TA GC	A CG GC GACCANA AG G GA T GG C TT ATC
	6 00	650
Nouse	GGTCGGGCGCCGGCGGCTTTGGTGACTCTAGATAACCTCGG	HECCB-ATCOCACGECCCCCGTGBCCBCCBACBACCACCATCBAACGTCTGCCCTATCAACT
Yeast	C C C TAA TI	T - T - ANDE TAAT GT TT A T G TT- CT T GTT AT A TT
E.coli	AT T C A AT GA Á	
	780	750
Mouse	TTCGATGGTGGTCGCCGTGCCTACCATGGTGACCACGOGTG	MCGGGGAATCAGGTTCCAATCCGGAGAGAGGGGGGCCCTGAGAAACCGGCTACCACATCCAAG
Xenopus Yeast	AC TT T C A GATAGTG TT A A	A
E.coli	CATA GGTAACS TO TA C S TOCC -	GCT TC & A GAT COAGCC C CT GAA C TOOAG TC T C
	566	
Mouse	GAAGGCAGCAGGCGCGCAAATTACCCACT	
Xenopus		G
E.coli	G TG G A T G A A GGGCGCAAGCC1	GATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTA TTTCAG
	858	
House		
Xenopus		
E.coli	A G GT GT T CCTTT CT AT GA TT	GCAG AGAAGCACC G T AC CCETG AGC G CGC G AA
		I8S+ITS I
Ma		
Rat	GCTGAGAAGACGGTCGAACTTGACTATCTAGASGAAGTAAA	MGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAACGGGAAGACTGTGG
Xenopus Yeast	C A A G TTT A A GTC T G C	ТС А СС СССТС
	•	
Nouse Rat	AGGAGGGG	GASTTC-SCTCACACCCGAAAATACCGATACGACTCTTASCGSTGGATCACTCGSCTCST
Xenopus Yeast		A CCGA-C GACGCGT GCGAGAGCTCC
	2050	2100
Mouse Rat	GCGTCGATGAAGAACGCAGCTAGCTGCGAGAATTAATGTGA	MTTGCAGGACACATTGA-TCATCGACACTTCGAACGCAC-TTGCGGCCCCGGGTTCCTC
Xenopus Yeast	A G AA T C9	A TT CG A AT T A C TT ATTC
		. 3160
House Rat	CCGGGGCTACGCCTGTCTGAGCGTCGCTT_GACGATCAATC	
Xenopus Ye as t	С 6 CC TC - ме с-т т мт ссттстся си	
	ITC 24200	
	115 LY205 3200	3259
Mouse Rat	TCCCCCCTTTCTEACCCCCACACCTCAGATCAGACCTGCCGA	CCCCCTCAATTTAACCATATTACTCACCOCACCAAAAAAAA
Xenopus Yeast	C CCCAC TCA C CAATGTTC AAA TTT A GTAGGAGT	C A C C C A A C C C A A C C C A A C C C A A C C C A C C C A C
Moure		
Xenopus	e e	



FIG. 4. Location and possible structure of the insertions found in mouse and X. laevis 18S rDNA as compared with yeast 18S rDNA. The vertical arrows indicate the borders of homologous sequences, at which inserts occur in higher organisms. Asterisks denote the actual inserted nucleotides. Numbering corresponds to the sequence in Fig. 2.

end of 5.8S rRNA and possibly for 4 to 7 nucleotides after the 3' end of 18S rRNA (Fig. 3). In addition, all three vertebrate species have a block of pyrimidines 5 to 15 nucleotides upstream from the 5' terminus of 28S rRNA, and all the eucaryotes show at least five additional purines beyond the 3' terminus of 18S rRNA.

Analyses of possible secondary structure. Possible dyad symmetries near the ends of mature rRNA termini and in the adjoining spacers were determined by computer-assisted analysis. The most stable structures ($\Delta G < -15$ kcal) are represented in a matrix (Fig. 7) for the available sequences of X. *laevis* and mice. Hairpin loops are located along the diagonal, indicating base pairing between nearby pre-rRNA sequences. Larger loops arising from possible long-range base pairing appear at appropriate distances from the diagonal. For each loop, the thickness of the enclosing oval corresponds to the ΔG^0 of the base-paired stem (see legend to Fig. 7).

Amid the complexity of the graphs, some features stand out. First, the potential for structure formation is very great within each spacer region, and many stable base pairings are possible, even between ITS 1 and ITS 2. Nearly every stretch of 20 nucleotides can potentially match with at least one other stretch, and some short regions show many possible matches with sequences scattered throughout the pre-rRNA.

Second, the capacity for the formation of stable secondary structure is far lower in mature rRNA sequences. These regional differences in the potential for secondary structure are most extreme in the X. laevis matrix, in which the complete 18S rRNA sequence can be compared with three spacer regions.

Third, the regions with a high density of potential secondary structure approach, but do not usually include, the termini of certain mature rRNA species. In fact, windows of sequence with far less capacity to form dyads extend up to 60 nucleotides from the termini into spacer regions. In some cases, the border of a window is very well defined, e.g., the spacer sequence proximal to the 5' end of 5.8S rRNA (Fig. 7). The same windows appear in the matrix analyses of rat rDNA (data not shown).

Fourth, no dyad symmetries are observed with the stability, length, or relative position of

FIG. 3. Alignment of rDNA sequences corresponding to mature rRNA of different species. Mouse rDNA is compared with available sequences of rats, X. laevis, and Saccharomyces cerevisiae (ITS sequences from Saccharomyces carlsbergenesis); an alignment to E. coli 16S rRNA is also included for the 5' portion of mouse 18S rRNA. Deletions (dashed lines) have been introduced in each sequence to maximize homology. The mouse rDNA sequence is presented in the top row, and numbers relate directly to the nucleotide sequence number in Fig. 2. Nucleotides that differ from the mouse rDNA sequence are indicated in the corresponding rows for each species, and spaces represent complete identity with the mouse sequence. Dots indicate absence of sequence data for a given region.

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Mouse

FIG. 5. Matrix comparison of mouse and rat rDNA sequences spanning the ITSs. (A) Each line indicates a region of homology of at least 20 out of 30 bases identical. Homologies appearing close to the diagonal are those that also correspond closely in relative location within the two sequences. (B) Bars represent corrected percentage of divergence (see text) between aligned regions of the mouse and rat rDNAs. The coordinates for sequence location match those in A, and each bar corresponds to a line of mouse rDNA sequence from Fig. 5 (mature rRNA sequences are represented by separate bars). Lines a through d show the location of mouse rDNA regions corresponding to those of rat spacer rDNA that have relatively high homology to X. *laevis* rDNA (29).

those that form stems to enclose 16S and 23S rRNA sequences in *E. coli* pre-rRNA (4).

DISCUSSION

A curious feature of phylogeny, namely the progressive increase in length and G+C content of mature and spacer rRNA, can now be exam-

ined by comparative sequence analysis. One can also assess whether the evolution of any spacer sequences is constrained in a way which implies functions in rRNA metabolism.

Evolution of mature rDNA sequences. Sequence comparisons of procaryotic rDNAs indicate that the primary sequences of rRNA are highly conserved. *E. coli* and *Bacillus brevis*

FIG. 6. Computer-assisted alignment of mouse (M) and rat (R) rDNA sequences spanning ITS 1, ITS 2, and adjacent mature rRNAs. Asterisks indicate homologous bases; gaps in both sequences have been introduced to maximize homology.

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		COLTONGARGAC GECGARCTEGACTATETAGAGGARGTANANGTEGTANCA GGTTECCGTAGGTGANCCTGEGGANGGATCATTACCGGANGGATCATTACGGANGGAC	1 66 97	18 S

	Μ	GAUGAGEG GEGEGETUGECCUCTETECCEGTETTGTGTGT GTECTEGEEGGGGGGGGGGG	189	
	R	GA060000TCGT0CCCGTCCCCTTT000CCTGTGT0AUTGTTCCTCCTCTCGCC00GA0GC0CGTCCCC00GT00GTCCCCGTGTCCC00CGTCCT00C	197	

	N	AGCGAGGTGTCTGGAGTG AGGTGAGAAAAGGGGTGGGTGGGTGGGGTCGGTCCGGTC	265	
	R	GTCGTGGCGCGTGCCGTGGCCGGCCGGCGGAGGGGGGTTTCGGGACAGGTGTGCGGGTGTGGGGGTGTCGGGTCTGGTCCGCCGCGGGGACCTCCTCCGTTTT	297	
		*		
	N	cccctcccctcccctctccctcgtccggctctancctcgccacctnccgggcggcggctgctgcggggggtgctgctcttgcctcttttcccgtccggctgct	365	
	R	CTCGCTCTTCCCTCGACGCCTCCGT GCGCCCGCCTCCGCCGCCGCCGCGGCAGCGA GGGCGTTCTGCCTCTTTCCCGACCGGCCCGCCCGC	392	
		*		
	м	CGTGTCTACG AGGGGCGGTACGTCGTTACGGGTTTTTGACCCGTCCCGGGGGCGTTCGGTCGTCGGGGCGCGCGC	463	
	R	\$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$	492	
		+		
	м		557	
	P		583	
				ITS
	м		654	
	p			
	~			
	~		725	
	ĸ	TELECOUR TELEVENUE TELEV	781	

	M		829	
	R	G COCACCCCTCTOCCOCCOGCOCTTCCCCAAOGCOGCOGTTC GOOGOOGCGTOTCBCCAAOGCCCCCTCTOGGCOCCCCAAAOGOTTCCCCGAC	871	
	M	CCGCGCCGCCGGCTGCCGATTTCCGCGGGTCGGT CCTGTCGGTGCCGG TCGTGGTTCCCGTGTC GTTCCCGTGTTTTTCCGCTCCCGACC	911	
	R	CCGMGCGCCTGGCTTGTCCCGCCCGTTGGCGGGCCGCCGCCGGGTGCCGGGTGCCGGGTTCGTGGCGTCCCGTGTCCCGTGTGTCCCG CCTTTCCGTCTCCGACC	161	
	M	CTITITITITCCTCCCCCCCAC ACGTGTCTCGTTTCGTTCGTTGCTGGCCTGACGCTGACGC TACCCCTCOGTCCATCTGTT CTCCTCTCT	1002	
	R	CTGTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	1060	
		+		
	N	TCC0000ANGA000CCGTGGTCGTT00000ACTGT0CCGTCGTCA0CACCC GTGAGTTC GCTCACACCCCBAAATACCGATACGACTCTTAG	1095	
	R	OCGCAGGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	1161	
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	N	CG01GGATCACTCGGCTCGTGCGTCGATGAAGAACGCAGCTAGCT		
	R		1195	585
			1201	
	м	TOCOOCCCCOOLTICCTCCCOOLTICCTOTCTOTCTOCCTTOCCOTCCCTTOCCOTCCCTCCC		
	R		1276	
	w	TGCGCGGCT6GGAGTTTGCTCGCAGGCCCAACCCCCCAACCCCCGGTCGGGTCGCGCCCCCCGTCGGGGCGCCCCGGCGG		
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	M R	TGCGCGGCTGGGAGTTTGCTCGCAGGGCCCACCCCCCGGGCCGGGCCCTCCGTCTCCCGAAGTTCAGACGTGTGGGGCGGTTGCGGCG CGCGCGCTGGGAGTCTGCTGCCGGCGCCGCCCCCCG CGCGCGCCGCGGGGCCCGCCC	1 376 1448	
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			1376 1482 1482 1582 1582 1586 1595 1595 1597 15917 18917 18917 18915 15915	ITS 2
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CORCECTCCCCCCORCECTCTCORRECCCCCTINALACONTCRCCORC TCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC			1376 1442 1482 1580 1580 1580 1580 1580 1580 1596 1596 1997 1844 2015 1844	ITS 2
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			1376 1443 1443 1583 1583 1583 1583 1584 1585 1581 1917 1917 1917 1917 1917 1917 1917 19	ITS 2
COCTCTCTTCTTCCCCCCGGCTGGGCCGCGTGGGCGCGCCCTCTTTCTGGCGCGCCCCCC			1376 1442 1482 1389 1389 1389 1589 1589 1596 1596 1596 1997 1887 1777 1884 2015 1941 2115 1941	ITS 2
CC68TCGCCCG6C05CGCCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCACCCCCC			1376 1489 1489 1589 1589 1589 1589 1589 1589 1589 15	ITS 2
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			1376 1443 1443 1523 1523 1524 1535 1541 1777 1775 1917 1917 1917 1917 1917 191	ITS 2 285



FIG. 7. Matrix representation of possible secondary structure in mouse (A) and X. laevis (B) pre-rRNA. Each matrix indicates the location of possible dyad symmetries based on a computer search for base pairing sequences. Finest gradations on each axis represent distances of 25 nucleotides. Lines within ovals correspond to the sequence coordinates and length of each strand in a base-paired stem; the surrounding oval indicates relative stability of the structure. The stability (ΔG^0) of each possible stem is calculated according to the rules of Tinoco et al. (30, 31) and the free energy calculations of Borer et al. (1). Thinnest oval = lowest stability range: $-25 \text{ kcal} < \Delta G^0 \leq -15 \text{ kcal}$. Middle range: $-35 \text{ kcal} < \Delta G^0 \leq -25 \text{ kcal}$. Highest range: $\Delta G^0 \leq -35 \text{ kcal}$. Points near the diagonal indicate the smallest (hairpin) loops between closely spaced sequences. The mouse rDNA matrix has a dotted line denoting a gap in the available sequence data for 18S rRNA. Conditions for dyad symmetry search: homology of $\geq 70\%$; ≥ 7 bases paired; loopouts within the stem limited to single base bulges; any mismatches within the stem must be followed by ≥ 3 matched base pairs; G-U pairing is allowed.

rDNAs have long, nearly identical regions with an occasional sequence insertion or deletion (37). The extra or missing sequences in one or another species appear to be within largely selfcontained regions dominated by short-range structure. Models of procaryotic rRNA secondary structure also show considerable overall conservation (27, 28, 37, 39). These observations are consistent with the similar function and common antibiotic sensitivity of ribosomes from many bacterial sources (32).

Analogous to the similarities among procaryotic rRNAs, antibiotic sensitivities and functional characteristics of yeast ribosomes are largely the same as those of mammalian ribosomes (32), and the mature rRNA sequences have corre-



FIG. 7. Continued.

spondingly high homology. Some of the base substitutions from yeasts to X. laevis to mice (Fig. 3) may be compensatory base changes (see also reference 24). Even the G+C-rich inserts within the 18S rRNA sequences of mice and X. laevis probably form stable hairpin stems (Fig. 4) and may not disturb the structure of neighboring segments.

These G+C-rich inserts are quite unlike the introns observed in genes coding for proteins since they are not removed from the RNA by processing. The insertion of these elements can account for at least part of the progressive lengthening of mature rRNA during evolution as well as the trend toward higher G+C content. For example, the first 595 nucleotides of 18S rRNA from yeast, X. laevis, and mice show G+C contents of 40.9, 52.1, and 56.2%, respectively.

The rate of evolution of mature rRNA sequences can be estimated from the corrected percentage of divergence (calculated from pairwise comparison of the aligned mouse, X. laevis, and yeast sequences) and from the approximate dates of divergence of these organisms. A 1% change in the nucleotide sequence of the mature rRNAs (the UEP) takes 30×10^6 to 100×10^6 years. The divergence of mature rRNAs is much slower than that of protein-encoding genes like globin (from DNA sequence data: UEP; $10 \times$ 10⁶ years) (12) or preproinsulin (from DNA sequences: UEP, 5×10^6 to 25×10^6 years) (20). Although change occurs faster than in histone H4 (from protein sequences: UEP, 400×10^6 years) (36), the mature rRNA sequences are among the most highly conserved that have been studied.

Evolution and function of spacer sequences.

Two trends are apparent in the evolution of eucaryotic transcribed spacer regions. Analogous to the expansion of mature rRNAs, the length of spacers increases progressively from yeast to X. *laevis* to mouse (363 to 561 to 998 nucleotides for ITS 1; 235 to 263 to 1,018 nucleotides for ITS 2). Second, the proportion of G+C is much higher in vertebrate spacers than in yeast: for example, the yeast ITS 1 contains 35.2% G+C, compared with 83.9% in X. *laevis* and 70.1% in mouse.

One mechanism for the expansion of the spacers may involve repeated insertion of small G+C-rich sequences. The frequency of these events is apparent in the comparison of mouse and rat ITSs (Fig. 6), in which the optimized alignment contains 28 deletions and 25 insertions in the mouse spacer. Although rare deletions or insertions are as large as 120 nucleotides, nearly all are in the range of 4 to 40 nucleotides. Thus, small regions of the spacers may be continually deleted, and as in mature rRNA, small G+Crich segments may be inserted. However, it is also possible that the rare insertion of large segments (see ITS 2, Fig. 6) may be the major mechanism for the expansion of spacer sequences during evolution.

The rate of insertion (greater than 3 nucleotides) in the mouse versus the rat ITSs is about 1.5 insertions per 100 nucleotides per $100 \times 10^{\circ}$ years. The corresponding rate for insertions into the first 500 nucleotides of mature 18S rRNA for mouse versus X. laevis or X. laevis versus yeast is about 10-fold lower (0.05 to 0.16 per 100 nucleotides per 100×10^6 years). The slower accumulation of insertions in the mature sequences is consistent with the slower increase in length of mature versus spacer sequences. It could reflect additional mechanisms for variation in the spacer sequence; alternatively, the rates of insertion might be comparable, but many insertions into mature rRNA could be lethal or disadvantageous.

The mechanism by which these G+C-rich insertions are generated can only be the object of speculation. Zea mays chloroplast 23S rRNA contains insertions compared with E. coli 23S rRNA (11). Many of these insertions are flanked by short direct repeats and contain inverted repeats. Similarly, the insertion into the mouse sequence at nucleotides 574 through 596 is flanked by a direct repeat (nucleotides 565 through 573 and 613 through 620) and contains an inverted repeat (see Fig. 3 and 4). Because flanking direct repeats and internal inverted repeats are characteristic of bacterial insertion sequences (6), it has been proposed that the Z. mays insertions are insertion sequence-like elements (11). Another possibility is that the insertions were generated by slippage of the two DNA strands during DNA replication (12). This mechanism has been suggested for the insertion or deletion of sequences near direct or inverted repeats. However, the sequences surrounding the mouse inserts and the inserts themselves are not entirely consistent with any present model for the generation of deletions or insertions. This may be due to divergence of these sequences after the insertion event.

The comparison of mouse and rat ITSs also demonstrates that base substitutions occur at a high rate within these sequences. The overall rate corresponds to a UEP of 0.3×10^6 years. This is comparable to the UEPs of 1×10^6 to $6 \times$ 10^6 years calculated for base changes within noncoding regions, 5' flanking regions, and intron sequences in human β -like globin genes (12). We conclude that the drift of internal spacer sequences is largely dispersive and at least as fast as the divergence rate of any other gene sequences analyzed. This suggests that the vast majority of the ITS has no functions strictly dependent upon its primary sequence.

The one feature of the spacer sequences that is highly conserved among vertebrates is their high G+C content. One speculation is that the structure or replication of rDNA involves a modified conformation (38) that is dependent upon high G+C DNA. The high G+C content could also be important for the formation of stems and loops within spacer sequences. Interestingly, transcribed spacers from yeast and other lower eucaryotes contain a high percentage of A+T instead of G+C. Perhaps the small size of these spacers (as compared with those in vertebrates) obviates the need for high G+CDNA, or perhaps the high A+T content favors a structure analogous to that favored by high G+C content.

Evolution of sequences near mature rRNA termini. Of any regions within the transcribed spacers, sequences bordering mature rRNA termini seem most likely to function during rRNA metabolism. Therefore, flanking spacer regions were searched for evolutionarily conserved features. Short stretches of sequence conservation near mature rRNAs have already been mentioned. Another type of evolutionary consistency is a conserved pattern of repeats near the mature rRNA termini (Fig. 8). Direct 4-nucleotide repeats are found at the 3' ends of 18S and 5.8S rRNAs from mouse, X. laevis, yeast, and rat (all perfect repeats except near the rat 18S terminus). These repeats have different sequences in different organisms but are located at nearly identical positions. Less suggestive imperfect repeats of 5 to 7 nucleotides are seen at the 5' ends of 18S, 5.8S, and 28S, rRNA in all four species, always with a single nucleotide stutter (e.g., ABCD . . ., ABXCD . . .). The Vol. 3, 1983

MOUSE rDNA SEQUENCES 1499

Heuse 185

Rat 105 CCBCCBCBTCTCGCCTCGCCGCTCCTTACCTBCCTBATCCTBCCCBATAGCATATECT

Xenopus 198

Yeast 105

5.85

NETTCECTCACACCCEANA<u>ATACCEATACEACTCTTAECESTEEATCACTCESEC</u>

TTCCBCTCACACCTCAGATAAC<u>CETACGAC</u>TCTTAGCGGTGGATCACTCGGCTCGTG

ACCOACOCG<u>TCGOCGA</u>G

TTTTCGTAACTGGAAATTTTAAAATATTA

Xeneeus 5.88

Yeast 5.88

OCTCOCONCTCTTAGCOSTOGATCACTCOSCTCOTO

MACTTTCAACAACSGATCTCTT66TTCTC6

House 144

TTCCSTASSTSAACCTSCSSAASSATCATTAACSSSAGACTSTSSASSASCSSCSTSS

Ret 185

Xenopus 188

TTCCSTARSTRAACCTCCBBAARSATCATTAACBAB<u>ACCC</u>CCCTC<u>ACCC</u>BBABABABBBBAA

YE 451 100

House 5.00

TCCC8866CTAC6CCT6TCT6A6C6TC6CTT6AC6ATCAATC6C8TCACCC6CT6C66T66

Rat 5.85

Xenepus 5.85

Yeast 5.85

TCCAREGORICATOCCTETTTEASCETCATTCCTTCTCAAAACATTCTETTTOETASTEAST

House 205

Rat 205

Xenopus 205

Yeast 258

FIG. 8. Short repeated sequences near mature rRNA termini. Perfect repeats are associated with 3' termini of each mature rRNA species; imperfect repeats are found near 5' ends. Only those repeats that are the longest and show some evolutionary consistency in relative location are indicated here.

evolutionary conservation of these repeats suggests that they may play some role in ribosome formation or pre-rRNA processing (2).

Our analysis also suggests that pre-rRNA is composed of two types of evolutionarily conserved structural domains. The first is characterized by an enormous potential for secondary structure within and between transcribed spacers (Fig. 7). These sequences may be knotted into very stable stems, as suggested by visualization of pre-rRNA structure by electron microscopy (35). The mature rRNA sequences and the regions 10 to 50 nucleotides from their termini seem to represent another type of structural domain without much possibility for the formation of stable stems. The boundaries of these two domains are fairly sharp and are found in all vertebrate pre-rRNA sequences analyzed (rat data not shown). Of the thousands of possible dyad symmetries distributed throughout both domains, only a few involve base pairing with sequence at a mature rRNA terminus. These stems are not conserved in sequence or location and therefore are not analogous. Thus, the mechanism of rRNA processing in mammalian cells may be very different from the bacterial paradigm and is considered further in the accompanying paper (3).

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