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Fine structure of ribosomal RNA. IV. Extraordinary evolutionary conservation in sequences that flank introns in rDNA*

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ABSTRACT

By hybridization and DNA sequencing, we have defined a specific region in Xenopus rDNA that is extremely conserved between Tetrahymena, a protozoan, and Xenopus, a vertebrate. This highly conserved region is found at the site where an intron has been shown to interrupt Tetrahymena rDNA [1,2], although we have not detected introns in genomic or cloned Xenopus rDNA. We have noted that the sequences corresponding to nuclear rDNA intron-flanking regions show an intriguing complementarity to $tRNA_1^{Met}$. This suggests possible models for tRNA-rRNA interactions in protein synthesis and/or rRNA splicing.

INTRODUCTION

The discovery that genes in eucaryotes contain intervening sequences, or introns, has altered our conception of DNA structure, RNA transcription, and the control of gene expression. Introns have been found in eucaryotic genes for rRNA, mRNA, and tRNA, that is, in gene classes transcribed by the three RNA polymerases. However, the significance of introns is far from clear.

The first genes discovered containing introns were those coding for ribosomal RNA (rRNA) in Drosophila [3-6]. Since then introns in nuclear rDNA cistrons have been identified in other Drosophila species [7,8]; other insects, namely Calliphora [9], and Sciara [R. Renkawitz-Pohl, L. Matsumoto, and S. Gerbi, manuscript in preparation]; in the protists Tetrahymena [1,2] and Physarum [10,11]; in the mitochondrial rDNA genes of yeast [12,13] and Neurospora [14,15]; and in the chloroplast rDNA of Chlamydomonas [16]. In some strains of Tetrahymena, all copies of rDNA contain an intron [1,2], suggesting that these $intron^+$ genes must be functionally active. In this case, a precursor rRNA has been found whose intron is subsequently removed in an early splicing event [17,18]. The situation is less well resolved in Drosophila where only about half the repeated rDNA cistrons contain introns, and the introns may only rarely be transcribed [19-22]. In this case, then, the $intron^+$ genes may not be functionally active.

In genes that code for mRNA, the sequences at the edges of the intron show similarities to one another [23,24], suggesting a role for these regions in the splicing reaction. However, the intron sequences adjacent to splice points in tRNAs [25], in organellar rDNAs [26,27], and in nuclear rDNAs [28, 60] do not share the sequences which are seen at mRNA intron boundaries; this suggests that different mechanisms are used for splicing in the three RNA classes. On the other hand, the position of the intron is relatively constant among eucaryotic nuclear rDNAs. This pattern of conservation of the location of the intron, but lack of conservation of sequence within the intron, closely parallels the situation found in different tRNAs and in mRNAs coding for the same protein in different species. For these reasons, we wanted to know whether the sequences flanking nuclear rDNA introns are conserved in diverse species.

Our hybridization studies have located regions conserved among the rDNAs of Xenopus laevis, distantly related eucaryotes, and a procaryote [29]. Some of these regions are in the general areas where introns are found in organellar and chromosomal rDNAs. Here we report that although we have been unable to detect introns in Xenopus rDNA, we have localized and sequenced a region of Xenopus rDNA that is extremely highly conserved with the regions flanking the intron splice point in Tetrahymena rDNA. This extraordinary evolutionary conservation suggests a role for this region in ribosome function and/or in rRNA processing. We present a model for the interaction of initiator $tRNA^{Met}$ with this region of rRNA during protein synthesis and/or in the splicing reaction.

MATERIALS AND METHODS

General

The origins and structure of the Xenopus laevis (amphibian), Saccharomyces cerevisiae (yeast), and Dictyostelium discoideum (slime mold) rDNA plasmids, growth conditions, DNA isolation, restriction enzyme sources and digestion conditions, transfer to DBM paper or to nitrocellulose, hybridization conditions, elution of fragments from gels, and electrophoresis conditions have been described previously [29]. Tetrahymena pigmentosa clones were generously provided by M. Wild and J. Gall [1], and Xenopus laevis clones were a gift from I. Dawid [30]. Restriction fragments of known length from pBR322 [31] were used as size standards.

DNA Sequencing

The Xenopus laevis rDNA clone pX1r11 was digested with Hinc II and the ends

were ^{32}P-labelled with polynucleotide kinase as described in ref. 32. After further digestion with Alu I, a fragment labelled at one 5' end from region r11-D (Fig. 1c) was isolated from a 5% acrylamide gel, eluted electrophoretically, purified by BND cellulose chromatography, and subjected to the DNA sequencing reactions described by Maxam & Gilbert [33,34]. For purines (A + G), the pyridinium formate reaction was used. The guanine (G) reaction utilized dimethylsulfate. Pyrimidines (C + T) were reacted with hydrazine, and the reaction for cytosine (C) alone used hydrazine in the presence of NaCl. Usually, an alkali reaction (A>C) was also performed. Gels of 10% acrylamide (Eastman Cat. #5521 is of low cost and is quite satisfactory), 0.3% bis-acrylamide (E.C. Corporation) were 40 to 65 cm long, 0.5 mm thick, and were run at 1500-2000 volts for varying lengths of time. The region of interest was verified by sequencing the complementary strand: an Alu I digest of fragment r11-D was kinase-labelled and then cut further with HgiA I plus Hae III to generate an easily separated 211 bp fragment labelled at one end (Fig. 1). Sequences were computer analyzed using the program developed by Korn et al. [35].

Preparation of Xenopus bulk DNA

Livers were removed from adult females of Xenopus laevis (Mogul-Ed, Oshkosh, Wisconsin), homogenized in 0.15M NaCl, 0.1M EDTA (pH8.0), and incubated with 0.5% Sarkosyl (ICN) and 100 µg/ml predigested Proteinase K for 30 minutes at 45°C. After sequential phenol and then chloroform: isoamyl alcohol extractions (24:1) and ethanol precipitation, the resuspended DNA was treated with RNAase A (50 µg/ml), RNAase T_1 (30 units/ml) and α amylase (100 units/ml), reextracted with chloroform: isoamyl alcohol, and the DNA was pelleted, banded in CsCl, and pelleted again as described previously [36]. The yield of DNA was about 1400 µg/liver.

RESULTS

By hybridization studies, we have previously mapped and identified restriction fragments of 50-300 bp in Xenopus rDNA within which there are at least 15-20 bp of continuous homology between Xenopus, Saccharomyces, Dictyostelium, and E. coli [29]. A partial restriction map of Xenopus rDNA is shown in Fig. 1. The expanded segment (Fig. 1c) is the Xenopus rDNA fragment showing the highest extent of conservation between the rDNAs of Xenopus and Saccharomyces as deduced from quantitative hybridization [29]. This region is about 2/3 of the way from the 5' end of 28S rDNA which

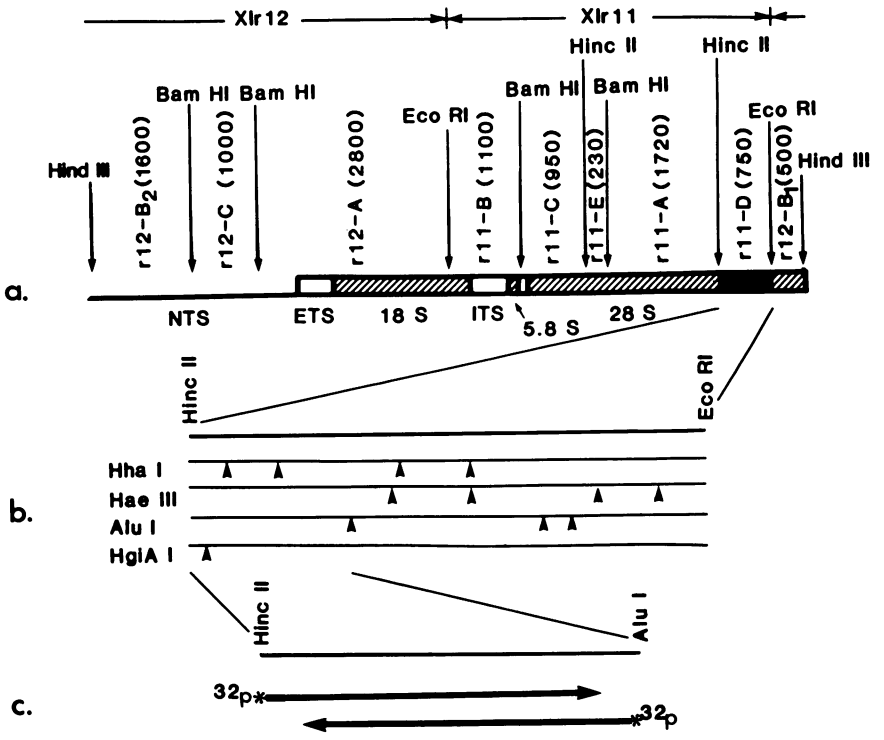

Fig. 1 (a) The Xenopus laevis rDNA repeat unit as represented in recombinant clones pXlrll and pXlr12 [29,37,38,39]; restriction sites defining fragments rll-A through E and r12-A through C are shown; sizes are in base pairs; NTS, ETS, and ITS are non-transcribed, external transcribed, and internal transcribed spacer, respectively; shaded regions refer to mature rRNAs; solid black region (fragment rll-D) is expanded in (b); restriction sites in rll-D referred to in this paper are indicated by arrow heads; (c) the portion of rll-D (237 base pairs) that was analyzed by DNA sequencing in this paper; the extent of the sequence determined on each strand is indicated by solid lines.

correlates with the position where introns in several organisms have been mapped using electron microscopy and restriction enzymes. By S1 digestion experiments using the method of Berk and Sharp [40], it was previously shown that Xenopus rDNA clone pXlrll does not contain an intron [37]. However, we reasoned that this extremely highly conserved region might be the site where introns are found in other rDNAs. Therefore we decided to investigate this hypothesis further by DNA sequencing.

It was convenient to define the section for sequence analysis by its homology to the intron-flanking region from Tetrahymena rDNA using the latter

as a probe (Fig. 2). In some strains of Tetrahymena pigmentosa, all 28S rDNA is interrupted by an intervening sequence, while in other strains all copies of rDNA are colinear with their mature rRNA product. The exact splice point in Tetrahymena rRNA has been mapped by Wild & Gall [1], and sequenced by Wild & Sommer [28]. The region in Xenopus rDNA homologous to the region surrounding the Tetrahymena splice point was identified in the following manner. Xenopus fragments r11-A through E and r12-A through C (see Fig. 1) were electrophoretically separated, transferred to nitrocellulose filters and hybridized with a 71 bp or 478 bp ^{32}P end-labelled Tetrahymena fragment containing the intron-flanking regions. (For a map of Tetrahymena rDNA indicating the fragments used as probes, see Fig. 2). Only r11-D showed hybridization (results not shown).

When this Xenopus r11-D fragment was digested further with Hha 1, electrophoresed, transferred to DBM paper, and hybridized to the same Tetrahymena probes, a Hha 1 fragment of approximately 70 bp hybridized strongly with the Tetrahymena probes (Fig. 2c). This 70 bp Xenopus fragment was also shown to be particularly highly conserved in hybridizations with yeast and Dictyostelium rDNA and rRNA [29].

To determine the exact extent of conservation between Xenopus and Tetrahymena in this region, this portion of Xenopus rDNA was sequenced

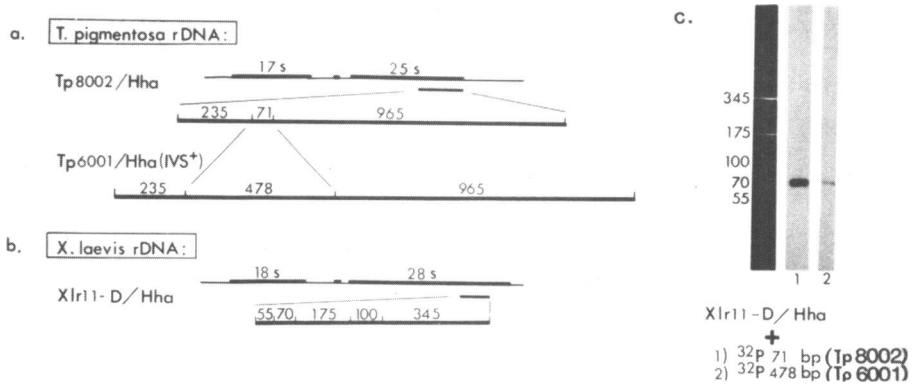

Fig. 2 (a) The Hha I restriction map of Tetrahymena pigmentosa rDNA clones as constructed by Wild & Gall [1]. Tp6001 is identical to Tp8002 except for the presence of an intervening sequence (IVS) of 407 bp [28]. (b) A map of fragment r11-D from Xenopus laevis rDNA digested with Hha I. (c) Results after separation of the Hha fragments of r11-D on a 7% acrylamide gel, transfer to DBM paper [41], and hybridization (1) to the ^{32}P end-labelled 71 bp (IVS$^-$) Tetrahymena fragment, or (2) to the 478 bp (IVS$^+$) fragment. A Xenopus fragment of about 70 bp shows positive hybridization.

using the method of Maxam & Gilbert [33,34]. The sequencing strategy is discussed in Methods. Fig. 3 shows representative gels from each DNA strand which include the regions of interest. The sequence is shown schematically in Fig. 4; the central 168 bp corresponding to either side of the Tetrahymena splice point (bases -60 to +108) were verified by sequencing both strands. In all, 237 bases of Xenopus rDNA sequence were determined: 92 on the 5' (left) side of the Tetrahymena splice point extending to the Hinc II site that defines the left end of fragment r11-D, and 145 bases on the 3' (right) side of the splice point extending to an Alu I site within fragment r11-D. Comparison of the Xenopus sequence with the Tetrahymena sequence showed that 221 of the 237 base pairs were conserved between the protozoan and the vertebrate, including 87 bp of exact identity spanning the Tetrahymena splice point. This is indeed remarkable since we had previously estimated that only 10-20% of rRNA as a whole is conserved between Xenopus and unicellular eucaryotes [29,36]. In fact, this region is even highly conserved with E. coli 23S rDNA. Comparison of the Xenopus 28S rDNA sequence with the E. coli sequence [42] (Fig. 4) revealed strong homologies in this region (E. coli bases 1885-2068), although no stretch is longer than 12 bp.

The extraordinary evolutionary conservation in this area encouraged us to look for introns in Xenopus rDNA. Morrow et al. [44], Brand & Gerbi [37], and Botchan and Reeder [45] investigated Xenopus recombinant rDNA clones for length heterogeneity in the 28S rDNA or lack of colinearity of mature rRNA and rDNA and did not detect any evidence of introns. We have extended this analysis to bulk chromosomal rDNA by looking for heterogeneity in the sizes of restriction fragments from genomic DNA detectable by hybridizations with radioactively-labelled restriction fragments from a Xenopus rDNA clone.

Hinc II+Eco Rl or Hind III+Eco Rl digests were performed on Xenopus bulk DNA. From restriction mapping reported earlier [29,38,39], these digests produce fragments coding for 28S rRNA of 2050 bp (fragments r11-B + C), 1950 bp (fragments r11-A + E), 750 bp (fragment r11-D), and 500 bp (fragment r12-B_1) (Fig. 5a). Hybridizations with ^{32}P-labelled probes from each of these regions (Fig. 5b & c) indicate that the sizes of the genomic rDNA fragments are the same sizes as those found in the recombinant DNA clones which lack introns (Lane 1:2050bp; Lane 2:1950bp; Lane 3:750bp; Lane 4:500bp). This suggests that introns, if they occur at all in Xenopus rDNA, are present in a very small number of cistrons. The unexpected 2700 bp band most likely

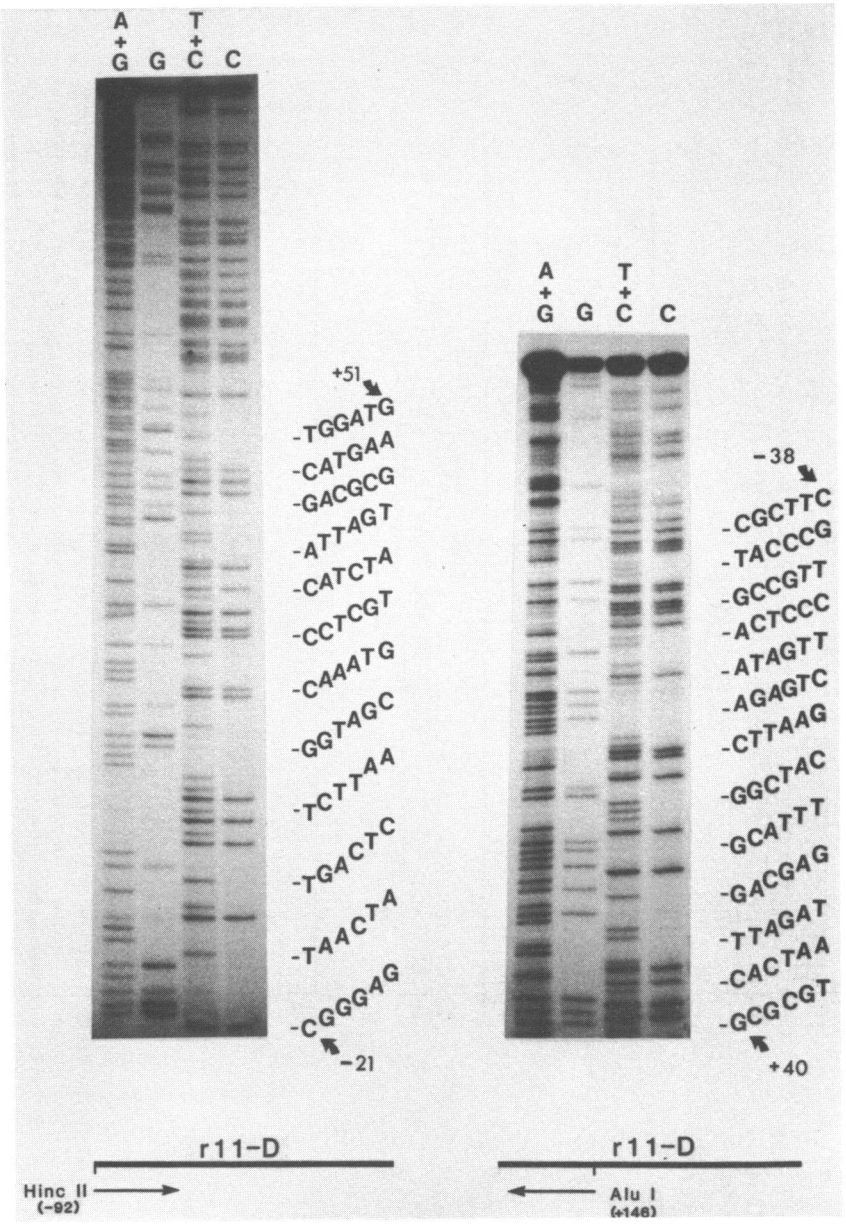

Fig. 3 Representative sequencing gels showing the sections from both strands of Xenopus rDNA corresponding to the Tetrahymena intron-flanking regions. Left: non-coding (rRNA-like) strand. Right: coding strand. The intron in the corresponding region of Tetrahymena rDNA is between positions -1 and +1.

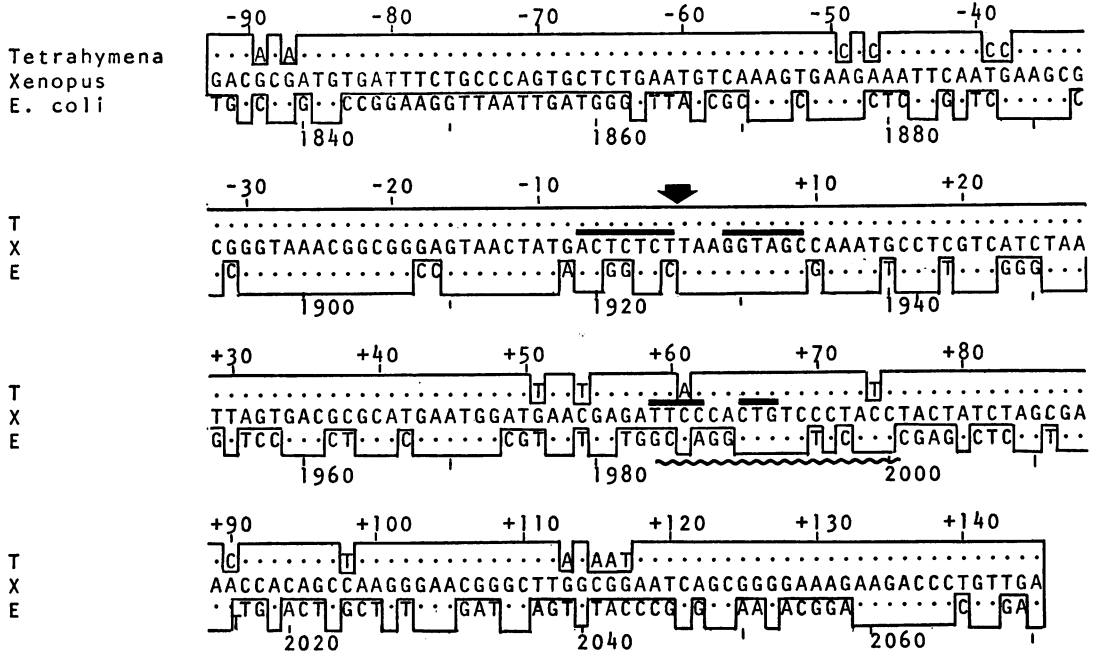

Fig. 4. Comparison of sequences from Tetrahymena [28], Xenopus (this paper), and E. coli [42]. Dots indicate bases homologous to the Xenopus sequence; arrow indicates position of intron in Tetrahymena; heavy lines indicate regions discussed in Fig. 6; wavy line indicates region in E. coli rRNA complementary to the D-loop section of E. coli $tRNA_f^{Met}$ [43]; numbering of Tetrahymena and Xenopus sequences is with respect to the position of the intron in Tetrahymena rDNA; numbering of E. coli rDNA is with respect to the 5' end of 23S rRNA. Only the non-coding (rRNA-like) strand is shown.

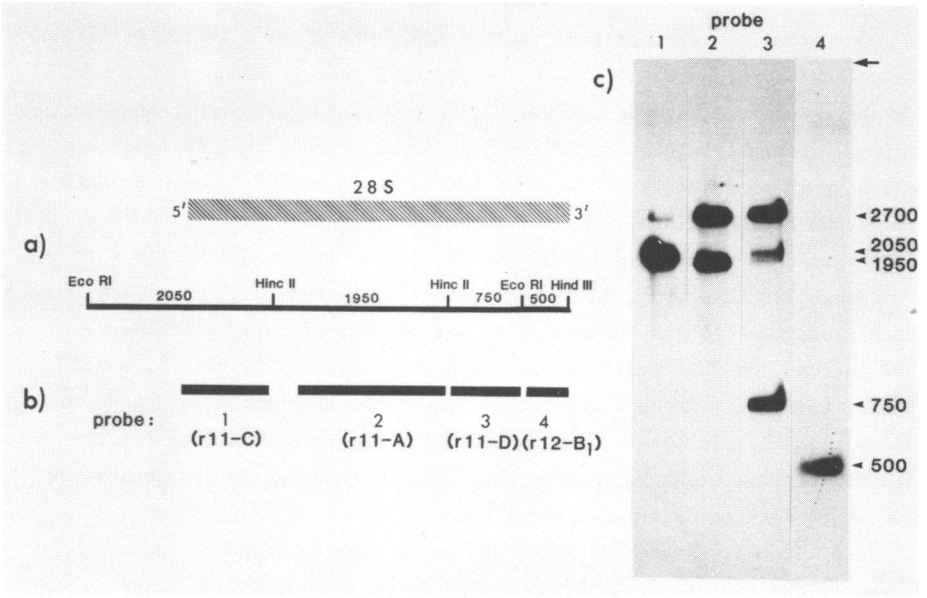

Fig. 5 (a) Structure of 28S region from recombinant clones of Xenopus rDNA (see Fig. 1). (b) Hybridization probes from Xenopus recombinant rDNA clones. (c) Hybridizations of radioactive cloned rDNA fragments to digests of genomic Xenopus DNA. Bulk Xenopus DNA was digested with Hinc II plus Eco Rl (Lanes 1-3) or Hind III plus Eco Rl (Lane 4), electrophoresed in wide lanes on 1.5% agarose gels, and transferred to a nitrocellulose filter. An arrow marks the origin. The filter was cut into strips and hybridized with cloned rDNA fragments #1-4 which had been ^{32}P-labelled by nick translation [29].

consists of rll-E + rll-A + rll-D (see Fig. 1), probably resulting from sequence heterogeneities, methylation [59] or partial digestion of the Hinc II site separating rll-A + rll-E from rll-D. The weaker bands in lanes 1,3 and 4 probably result from homologies and thus cross-hybridization between restriction fragments or (less likely) from cross-contamination in the probe preparations. We think it very unlikely that the 1950-2050 bp band in lane 3 results from cistrons containing introns, since this band corresponds exactly to the size of other rDNA fragments found in lanes 1 and 2 of this digest. If the 1950-2050 bp band in lane 3 were due to the presence of a 1200-1300 bp intron within rll-D, then one would also predict the presence of a band of 3900-4000bp resulting from the presence of this intron in the 2700 bp band. A band of greater than 2700 bp clearly is not found.

DISCUSSION

Our previous hybridization studies [29] identified a region about 2/3 of the way from the 5' end of the 28S rRNA gene in Xenopus that was strongly conserved between the rDNAs of Xenopus, Saccharomyces, and Dictyostelium. Several laboratories have identified introns in this same general area of nuclear rDNAs. Only the sequence of this region in Tetrahymena has been published [28]. Introns have not been identified in any vertebrate rDNA although splicing enzymes for at least some stable RNAs are thought to be present in Xenopus since yeast tRNAs can be spliced in Xenopus oocytes [46]. The positions but not the primary sequences of rDNA introns are relatively constant among eukaryotes [7,47]. It would appear, then, that the sequences that flank the rDNA intron might be the recognition signals determining the position of the intron itself. The flanking regions might be important because of their primary sequences or because of certain secondary or tertiary structures they can form.

Fig. 4 compares the sequence determined here for Xenopus rDNA with the corresponding regions of Tetrahymena and E. coli. The splice point in Tetrahymena rRNA is indicated with an arrow. It can be seen that 87 continuous base pairs of Xenopus rDNA sequence are identical to that of Tetrahymena (from positions -37 to +50); in total, the two sequences share 221 of 237 bp in common in this region. This represents 93% conservation between extremely divergent species. The homology between Xenopus and E. coli is also striking in the intron-flanking region. E. coli bases 1888 to 2068 and Xenopus bases -38 to +142 have several long nucleotide stretches in common and the distances between these stretches are of the same length in the two rDNAs.

As noted by Brosius et al. [42], the sequences flanking the yeast mitochondrial [27] and Chlamydomonas chloroplast [26] introns in rDNA are also highly conserved with E. coli 23S rRNA. However, these flanking sequences are found farther along towards the 3' end of 23S rDNA, at E. coli positions 2448 and 2593, respectively. We have also sequenced the Xenopus region corresponding to these positions (Gourse & Gerbi, manuscript in preparation). Xenopus and E. coli rDNAs are conserved in these regions as well, but the distinction between these regions and the region where the Tetrahymena intron is found is that the latter region is much more highly conserved among eucaryotes [29]. Thus, there are 3 distinct regions in E. coli 23S rDNA that show homology with eucaryotic intron-flanking regions: position 1925 with nuclear, 2448 with mitochondrial, and

2593 with chloroplast rDNA. The fact that all rDNA intron-flanking regions that have so far been sequenced show a high degree of conservation between eucaryotic and procaryotic rDNA suggests that the sites where introns appear are not random, i.e., that introns interrupt functionally important regions of rRNA. It has previously been speculated that introns in genes coding for mRNA serve to join genetic information for two distinct functional domains of the encoded protein [48]. A corollary of this is that the intron itself should be inserted in a region of DNA with little or no functional importance [49]. Although little is known about functional domains in rRNA, our data that the region bounding an rDNA intron is virtually indistinguishable between a protozoan and a vertebrate, suggests that the above corollary cannot apply for rDNA. That is, the conserved nature of this region suggests that it is likely to be of functional importance.

In this regard, we have noted that the sequences directly adjacent to the "splice point" on the Xenopus sequence show an intriguing complementarity to the acceptor arm of Xenopus initiator $tRNA^{Met}$. As diagrammed in Fig. 6a., (with the tRNA pictured in the clover leaf configuration for ease in showing base pairing), six bases at the 3' end (#70-75) and 7 bases at the 5' end (#1-8 with base #3 looped out) could conceivably base pair with the sequences -1 to -7 and +4 to +9 of the rRNA sequence. Such an interaction yields 13 paired bases which could be thermodynamically favored over the pairing between 7 bases otherwise present in the acceptor stem of the tRNA.

One might speculate that a primary role of this rRNA region is to pair with $tRNA_i^{Met}$ during initiation of protein synthesis. Although we have no direct evidence on this point, there is reason to believe that an interaction between initiator $tRNA^{Met}$ and the large subunit rRNA might occur during protein synthesis. In E. coli, affinity labelling studies suggest interaction between the 3' two-thirds of 23S rRNA and the aminoacyl end of $tRNA_f^{Met}$ (reviewed in ref. 58). Dahlberg et al. [43] have identified possible base pairing between E. coli 23S rRNA and $tRNA_f^{Met}$ involving bases 1984-2001 of the rRNA and the D-stem and loop section of the tRNA. In addition, this region of 23S is likely to be near the surface of the ribosome, available for interaction with other molecules, since E. coli 23S base 1954 is one of those modified by kethoxal [51].

In eucaryotes, there is less direct evidence for contact of 28S rRNA and $tRNA_i^{Met}$, but it should be noted that the binding of initiator $tRNA^{Met}$ to the 40S subunit is an obligatory step in the formation of 80S ribosomes (reviewed in ref. 61). In this case, $tRNA_i^{Met}$ is bound to 40S subunit complexes which subsequently become 80S initiation complexes.

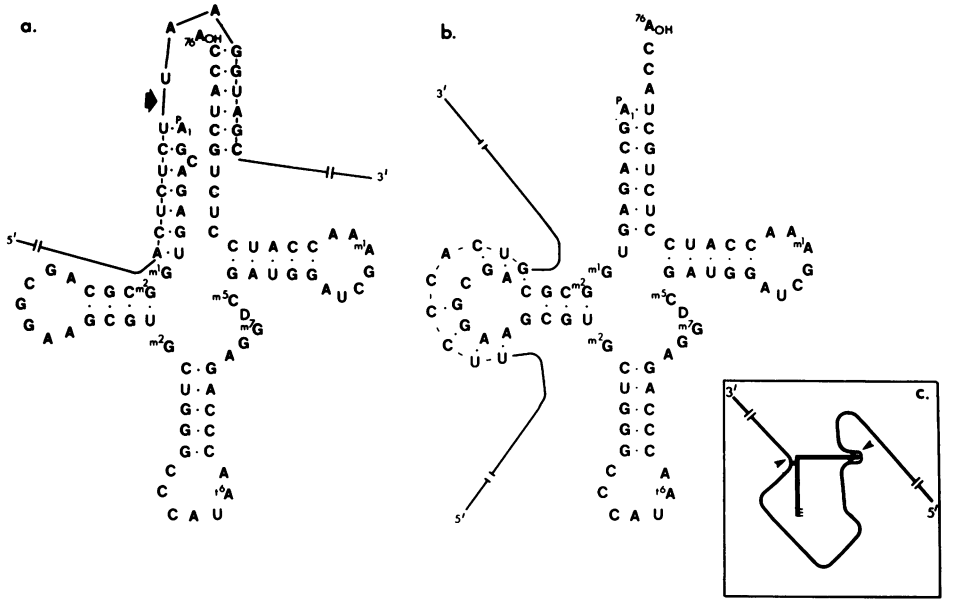

Fig. 6 Models for base pairing between Xenopus initiator $tRNA^{Met}$ and Xenopus rRNA. The tRNA sequence is from Wegnez et al [50]. The 5' base of the tRNA is #1, the 3' base of the tRNA is #76. (a) Possible base pairing involving Xenopus rRNA bases -7 to +9 (see Fig. 4) and the tRNA acceptor arm. An arrow indicates the position where an intron interrupts a corresponding section in the rRNA of Tetrahymena. (b) Possible base pairing involving Xenopus rRNA bases +59 to +67 (see Fig. 4) and the tRNA D-loop. An intron in a corresponding section of Drosophila virilis rRNA occurs about one dozen bases 3' (on the rRNA) of the possible tRNA (D-loop)- rRNA interaction. (c) Inset: schematic representation of the rRNA-tRNA interactions. The tertiary configuration of the tRNA is pictured schematically as an L. The interaction at the right of the inset represents the tRNA (acceptor arm) - rRNA base pairing; the nearby arrowhead indicates the position of the Tetrahymena intron. The interaction at the left of the inset represents the tRNA (D-loop)- rRNA base pairing; the nearby arrowhead indicates the position of the Drosophila virilis intron. It should be noted that the two rRNA-tRNA interactions need not occur simultaneously.

The association of the 60S subunit seems to result in the stabilization of bound $tRNA_i^{Met}$ [62]. Thus, it seems plausible that a 28S rRNA-$tRNA_i^{Met}$ interaction is involved in the formation of the initiation complex in eucaryotes.

There are seven possible Watson-Crick base pairs between the D-loop of Xenopus initiator $tRNA^{Met}$ and the region in Xenopus 28S rRNA (+59 to +67, see Fig. 6b) corresponding to the E. coli 23S rRNA region that base pairs

with E. coli $tRNA_f^{Met}$. Thus, the pairing possibilities in this region of Xenopus rRNA are not quite so extensive as those seen in E. coli. On the other hand, the pairing possibilities between E. coli 23S and $tRNA_f^{Met}$ in the region where the Tetrahymena intron is found are not so good as they are between Xenopus rRNA and tRNA. Nevertheless, it is possible that in both procaryotes and eucaryotes both sites in rRNA (see Fig. 4) make contact by base pairing with initiator tRNA.

We have recently learned [60], that an intron in nuclear rDNA from Drosophila virilis has been identified in the region corresponding to about one dozen bases 3' of where the Xenopus $tRNA_i^{Met}$ (D-loop) - 28S rRNA pairing is possible. In this case good base pairing possibilities exist in Drosophila rRNA with the D-loop region of Drosophila $tRNA_i^{Met}$. Thus, there seem to be two regions in rRNA separated by approximately 80 bases, with possible contacts to $tRNA_i^{Met}$. An intron in eucaryotic rDNA has been identified with each of these rRNA-tRNA contact regions. The inset in Fig. 6 presents an idealized diagram of this situation.

Therefore, we speculate that in organisms such as Tetrahymena in which introns evolved in nuclear rDNA, $tRNA_i^{Met}$ might have been utilized to play a second role (i.e., in addition to its primary role in protein synthesis), that of a "guide RNA" for splicing. Others [52-55] have suggested similar models in which small nuclear RNAs base pair with regions near the intron borders of mRNAs, aligning the flanking regions for splicing. In the latter cases, the proposed hydrogen bonding involves regions within the introns themselves rather than within exon regions as proposed here for the rRNA-tRNA interaction. In addition, RNA-RNA interactions have been implicated previously in stable RNA processing by RNase III and RNase P [56,57].

Our studies have demonstrated strong evolutionary conservation in a specific region of rDNA. The complementarity between rRNA and tRNA seen in this region may be important to ribosome function and/or rRNA splicing. It will be of interest to test this model by sequencing the nuclear rDNAs from organisms with different $tRNA_i^{Met}$ sequences in order to see if the possible tRNA-rRNA pairing is preserved.

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*Paper III in this series is reference 29.

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