TRANSCRIPTION OF MITOCHONDRIAL DNA

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I. INTRODUCTION

Mitochondria arise in the eukaryotic cell by growth and division of preexisting organelles and possess an autonomous genetic system. The coding capacity of mtDNA is limited and the majority of the proteins needed for mitochondrial biosynthesis are encoded in nuclear DNA, synthesized on cell-sap ribosomes, and imported into the organelle. The genes present in mtDNA include genes for components that function in the genetic expression of the mitochondrial genome, two ribosomal RNAs (rRNAs), and a complete set of transfer RNAs (tRNAs), together with genes coding for polypeptides that form part of the respiratory chain complexes in the inner mitochondrial membrane and a protein which is found associated with mitochondrial ribosomes. Both mitochondrial gene expression and the cooperation between the nuclear and mitochondrial genetic systems have been studied in a variety of organisms, but by far the most intensive research efforts have been concentrated on yeast and human mtDNA. On the one hand, yeast offers numerous advantages in terms of the application of refined genetic techniques to both nuclear and mitochondrial genomes. Human mtDNA, on the other hand, was the first mtDNA to be tackled by the newly developed DNA sequencing techniques and this, in conjunction with the painstaking analysis of human mitochondrial transcripts by Attardi and associates, has resulted in a wealth of information on a very remarkable genome.

When yeast and human mtDNAs are compared, we recognize the same basic set of genes (Table 1). The organization of those genes in both genomes is very different, however, and this has major consequences for the ways in which these genomes are expressed.

In this review we shall concentrate on mitochondrial transcription with the focus of interest on the human and yeast mitochondrial genomes. For further details on gene structure, organization and genetic code the reader is referred to recent reviews.[1-5]

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Table 1
MITOCHONDRIAL GENES IN YEAST AND HUMAN mtDNA

Mitochondrial component	Mitochondrial gene product in Yeast	Human
Cytochrome *c* oxidase		
Subunit I	+	+
Subunit II	+	+
Subunit III	+	+
Ubiquinol-cytochrome *c* reductase		
Apocytochrome *b*	+	+
ATPase complex		
Subunit 6	+	+
Subunit 9	+	−
RNA maturase		
*box*3 Maturase	+	− (?)
Large ribosomal subunit		
rRNA	+	+
Small ribosomal subunit		
rRNA	+	+
Ribosome-associated protein	+	− (?)
tRNAs	About 25	22
Unidentified Reading Frames[a]	⩾10	8

[a] URFs.

From Grivell, L. A., *Sci. Am.*, in press, 1982. With permission.

II. TRANSCRIPTION OF HUMAN mtDNA

A. Gene Organization

The complete nucleotide sequences of mtDNAs from man, cow,[3] and mouse[6] are now known and these, combined with the results of a detailed analysis of transcription in human mitochondria,[7,8] have revealed a mode of gene organization and expression of exquisite intricacy. The salient features are the most efficient with which genes are compressed into the available DNA, the contribution of genetic information from both DNA strands, and the use of tRNA genes to flank virtually every gene. None of the genes contain introns (Figure 1).

B. Symmetric Transcription

Perhaps the most radical departure from the behavior of more conventional genetic systems is the complete and symmetrical transcription of both strands of human mtDNA. The first indications for this unusual phenomenon were seen in the early transcription studies of HeLa cell mtDNA.[9-11] Whereas long-labeled RNA hybridized almost exclusively with the H-strand of mtDNA, RNA labeled in short pulses also hybridized with the L-strand. The ratio of hybridization to H- and L-strands approached 1.1 for RNA labeled with [^{3}H]uridine, a value expected for complete transcription of both strands, when the difference in dA content of each strand was taken into account. Consistent with this was the finding that self-annealing not only converted 60% of the pulse-labeled material to ribonuclease-resistant form, but also led to the appearance of long linear duplex molecules detectable by electron microscopy. The RNA synthesized on the L-strand is rapidly processed[9] and only eight stable tRNAs and one putative messenger RNA (mRNA) ultimately remain.[2] Transcription of the H-strand on which

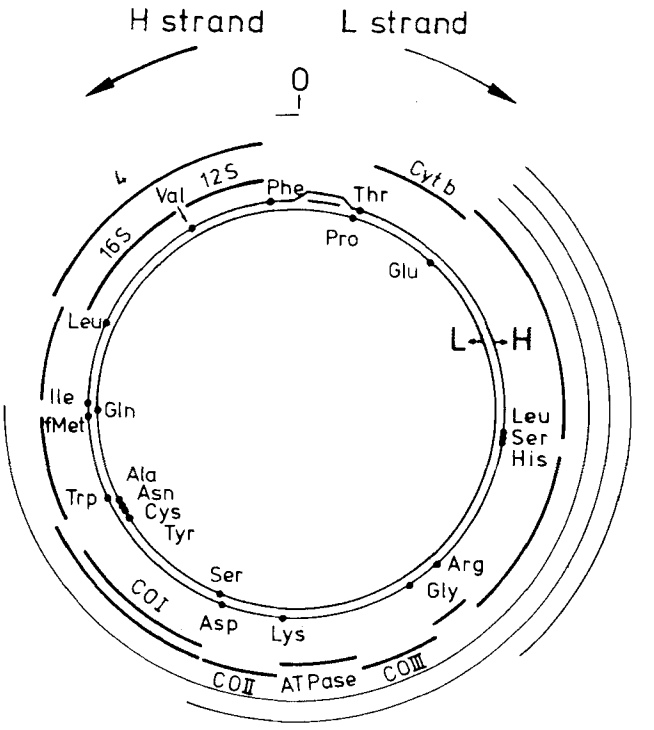


FIGURE 1. Transcription of human mtDNA. The figure was redrawn from data presented by Attardi et al.[19] O is origin of H-strand replication with the D-loop region on the right. Thin lines indicate L-strand transcripts, thick lines H-strand transcripts.

most of the genetic information is located gives rise to the 12S and 16S rRNAs, 14 tRNAs, and a number of RNAs of discrete size classes.[2] These RNAs lack a cap structure at the 5′-end,[12] as present on cell-sap mRNAs, they carry a poly(rA) tail of about 55 nucleotides at the 3′-end which is added posttranscriptionally,[13,14] and they can be isolated in association with polysomal structures.[15] Sequence analysis of both 5′- and 3′-ends has been carried out and most of these RNAs can be aligned with the known DNA sequence. Their perfect fit with known genes suggests that they represent mRNAs.[7,8]

The complete transcription of both DNA strands poses intriguing questions about the mechanism of transcription: how are RNA polymerases able to traverse the mtDNA from opposite directions without colliding? Why should the L-strand be expressed completely at an even higher rate than the H-strand even though its information content is minimal? How are complementary RNA molecules prevented from reannealing in vivo?

Some of these questions have been answered by Guiseppe Attardi and his group during their systematic and technically impressive studies on transcription and the metabolic behavior of mtRNAs in HeLa cells. The bulk of HeLa cell mtDNA is associated with the inner mitochondrial membrane, near the origin of replication. Treatment of such complexes with detergent (sodium dodecylsulphate = SDS) and pronase removes the membrane patch and associated proteins, but does not disrupt the association between nascent RNA strands and that fraction of the DNA that is transcriptionally active. The nascent RNA was purified from these complexes and examined for the presence of common ends by hybridization to labeled fragments of mtDNA, followed by treatment of the hybrids with S_1 nuclease. Common 5′-termini imply the existence of a common promoter while common 3′-termini suggest processing, pause, or termination points. The

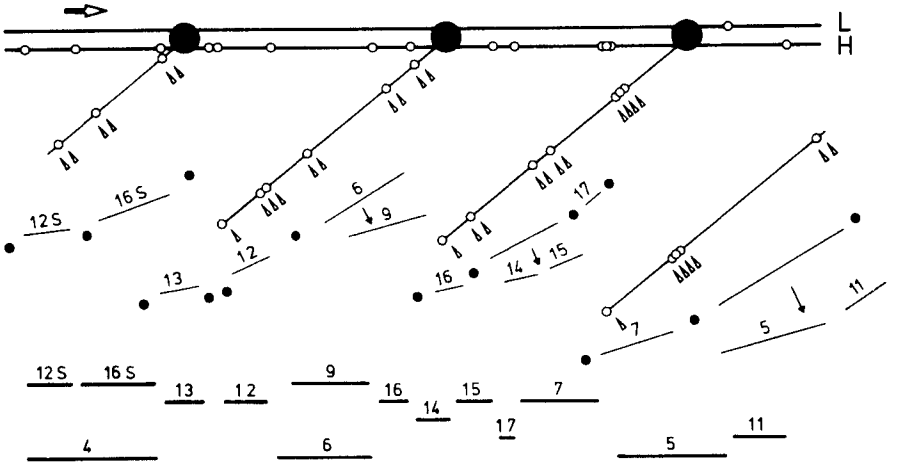


FIGURE 2. A model for the transcription of the H-strand of human mtDNA and the processing of transcripts. Large, solid circles represent RNA polymerase molecules. Small, open circles are tRNA genes, while small solid circles represent mature tRNAs. The arrowheads indicate the positions at which the nascent RNA is processed.

results indicate that hybrids formed with the L-strand are concentrated adjacent to the origin of replication in the direction of the L-strand transcription, while hybrids involving H-strands are found near the origin in the direction of H-strand transcription.[17] The interpretation of these experiments is that the H- and L-strands are transcribed from promoters located in the immediate vicinity of the origin of replication (D-loop region). Since DNA synthesis requires an RNA primer and the E(xtra) strand in the D-loop region also has a higher turnover number than is strictly necessary for DNA synthesis, RNA priming and initiation of transcription may be related processes.[6] It is likely that processing into discrete RNA components takes place on nascent transcripts (Figure 2).[8]

Table 2 shows that the steady-state concentrations of the various mtRNA species are different. For example, the concentration of 12S rRNA is 60 times higher than that of RNA 16, the most abundant mRNA.[8] Since the half-life of 12S rRNA is only two- to fivefold longer than of most mRNAs, a difference in the rate of synthesis must be responsible for the different steady-state concentrations. Consistent with this expectation, a 20- to 60-fold higher rate of synthesis for 12S rRNA with respect to mRNAs has, indeed, been found.[8] What is the reason for the difference in the rate of expression between rRNA genes and the remaining genes on the H-strand? One obvious possibility is that the idea of a single promoter on the H-strand is wrong and that two transcription units exist, one for the rRNA genes and one for the remaining tRNA and protein coding genes. This proposal requires, however, that the 3′-terminal segment of the 16S rRNA gene contain an initiation site for RNA synthesis. This is unlikely because there is not a single nucleotide between the 16S rRNA gene and the gene for $tRNA^{Leu}$ and, moreover, the metabolic behavior of RNA species 4 (see Table 2) argues against it. The map position of this RNA suggests that it is the precursor for the two rRNAs, but this is improbable, because its rate of synthesis is more than a factor 10 too low. Since RNA 4 is not on the main route for rRNA synthesis, it can only arise as part of a longer transcript, making the two-promoter model implausible. This leads Attardi to consider the transcription attenuation models[18,19] shown as A and B in Figure 3. Model A simply postulates a low frequency of read-through at a termination site at the 3′-end of the 16S rRNA gene. The presence of a short hairpin at this point, which is present in human, bovine, mouse, and rat mtDNAs (Dubin cited by Attardi[19]), could be involved in this stop. To account for

Table 2
METABOLIC PROPERTIES OF RNA SPECIES FROM HeLa CELL MITOCONDRIA

RNA species	Half life (min)	Number of molecules per cell	Number of molecules syn. per min per cell	Number of nucl. polymerized per min per cell ($\times 10^{-5}$)
16S	215	—		7
12S	208	34,000	265	7
−4	39	44	0.8	—
Rest of the H transcripts	80	664	8.6	1.3

Note: Data were taken from Attardi et al.[19] Data given for "rest of the H transcripts" are the average numbers for individual transcripts. no L-strand transcripts are indicated in this table.

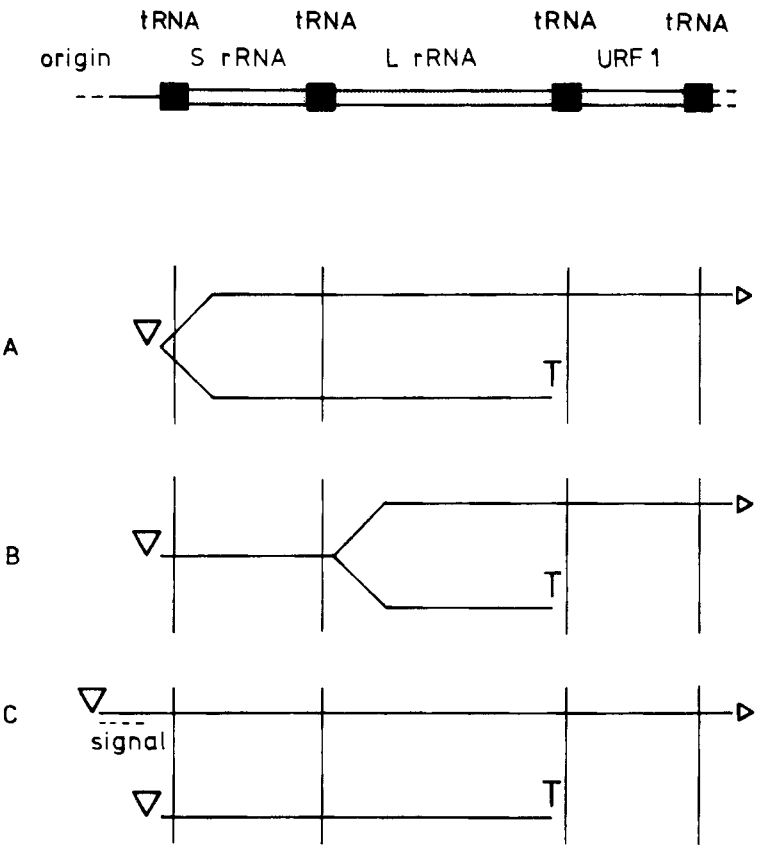


FIGURE 3. Regulation of rRNA and mRNA synthesis in human mitochondria. Part of the human mtDNA coding for the rRNA genes is represented. ∇, putative promoter; T, the position at which RNA can be terminated.

the low rate of synthesis of RNA 4, however, the fate of the transcript entering the 12S RNA gene has to be determined already. How this is accomplished in model A is not specified by Attardi.

In alternative B the fate of the RNA is determined by the $tRNA^{Val}$ gene locus between the genes for the two rRNAs. Here the rapidity with which the $tRNA^{Val}$ is processed out

of the nascent RNA strand influences what happens at the termination site: if the tRNA is taken out, the nascent RNA will change conformation and RNA polymerase will stop after transcribing the rRNA genes. This model is compatible with the slow synthesis of RNA 4.

The final possibility shown in model C is that transcription is initiated from two different promoters, both upstream of the rRNA genes. In this model it is easy to visualize how one transcript can have extra information necessary to determine its fate at the termination site compared with one that lacks this specific signal. For example, a ribosome binding site might function as such a signal. Ribosomes attached to such a binding site then could alter the secondary structure of the nascent RNA in such a way that the termination signal is ignored. A difference in strength between the two promoters could then explain the difference in the rate with which rRNAs are expressed compared with the remaining genes in the H-strand.

C. RNA Processing

The presence of a single promoter for each strand implies that mature tRNAs and mRNAs are produced by processing of a precursor RNA. The way in which almost every gene is flanked by tRNA genes[3,7,8] has led Attardi et al.[8] to propose a model of RNA processing, involving use of the tRNA sequences as recognition signals for cleavage of the polycistronic primary transcripts (Figure 2). Details of this process have yet to be worked out, but in the simplest version of the model it is assumed that the tRNA sequences fold into their cloverleaf structures, thus triggering precise cutting by processing enzymes which perhaps resemble the ribonuclease P of *Escherichia coli* in their mode of action. Although it is natural to assume that such processing enzymes have to be imported from the cytoplasm, it should not be forgotten that coding potential for up to eight proteins of unknown function is present in the form of unassigned reading frames (URFs) and it is possible that some of the URF products are involved in RNA processing. Alternatively, mitochondrial aminoacyl-tRNA synthetases could bind to their unprocessed substrates to provide the signals necessary for cleavage, thus providing a coupling between the rate of mitochondrial protein synthesis and that of RNA processing.

A curious aspect of the tRNA-punctuation model is that antisense tRNA sequences also must function as recognition signals, since the transcript containing sequences complementary to URF 6 and cytochrome *b* is processed at the position of the $tRNA^{Glu}$ which is encoded by the L-strand (Figure 1). Whether this particular sequence is capable of cloverleaf folding is not on record, but two observations suggest that the enzymes involved are not too exacting in this respect. The first is that all but one of the mitochondrial tRNAs has unconventional structures which fail to conform to the standard cloverleaf model.[20] Second, two genes — those for ATPase subunit 6 and cytochrome *c* oxidase subunit III — are not punctuated by a tRNA gene.[3,8] Nevertheless, the transcripts containing sequences of these genes (RNAs 14 and 15) get processed. Clayton et al.[6] have suggested that an alternative secondary structure consisting of a 31-nucleotide loop and an 8-nucleotide stem with the 5′-terminus of the COIII gene lying at the base of the stem on the 3′-side could replace the tRNA function in this case.

Although the transcription of the L-strand has proved much harder to analyze, early experiments suggested that this also is transcribed completely.[9-11] The L-strand transcripts described thus far do not span the entire L-strand,[8] however. They consist of three large RNAs with a common 5′-end point near the gene for $tRNA^{Glu}$ (Figure 1). They cover the URF 6 reading frame and all other L-strand encoded tRNAs with the exception of the $tRNA^{Pro}$, for which no precursor RNA has been found. A small RNA of unknown function maps in the region preceding the origin of replication. It seems likely that processing of L-strand transcripts is also dependent on tRNA punctuation. It may be that the very first precursor RNAs are present at too low concentrations to be detected and

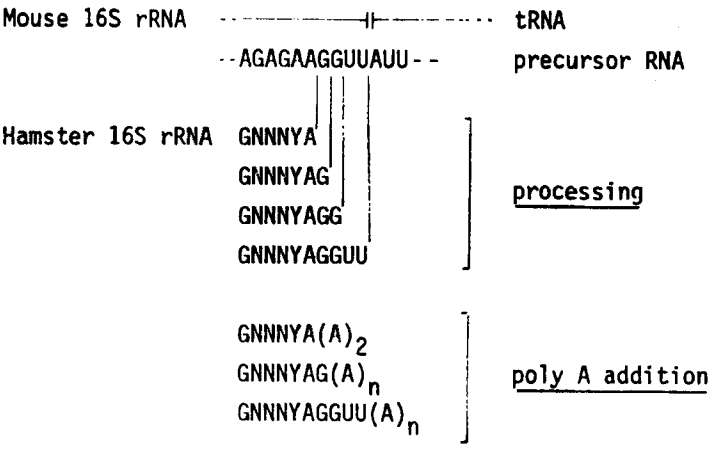


FIGURE 4. The ragged poly(A)-terminated ends of the small rRNA of hamster mitochondria. The upper line gives the RNA sequence for mouse 16S rRNA, which was used to align the ragged ends of hamster 16S rRNA.[21]

that the common 5'-ends of RNAs 1, 2, and 3 are due to processing from such an unidentified precursor. The 3'-ends of RNAs 1 and 2 correspond well with tRNA genes and that of RNA 3 also, but in this case the tRNA is that for Arg, located on the H-strand, demonstrating once more that the polarity of the tRNA structure in the RNA to be processed is of secondary importance.

D. Posttranscriptional Modification

Like many other eukaryotic mRNAs, those of HeLa cell mitochondria contain a poly(A) tail which is added posttranscriptionally.[13,14] Seven of the ten human mitochondrial mRNAs lack termination triplets and tailing plays an extremely unconventional role in maturation in that it turns the final U or UA in such mRNAs into a complete UAA stop codon.[3,8] Polyadenylation is not restricted to the mRNAs, however. RNA 4 is retained on oligo(dT)-cellulose[8] and sequence analysis reveals that even the two rRNAs receive extra A residues, which are not encoded by mtDNA (Figure 4). Mouse 12S rRNA has an extra 3'-terminal A^6 and hamster 16S rRNA has ragged ends terminated with a short poly(A) tail.[21] These observations have led to the consideration that the 3'-terminal A tailing of transcripts, alongside its possible function in translation, may also be implicated in some way in the processing of precursors.[8]

Polyadenylation is not the only posttranscriptional modification that mtRNAs can undergo. Inspection of the DNA sequence shows that the terminal CCA sequence characteristic for tRNA is not DNA encoded[3] and must be added later by a CCA-adding enzyme.

Mitochondrial rRNAs and tRNAs are only minimally modified by methylation compared with their cytosolic counterparts.[22-25] This is especially striking for methylation of the ribose (Table 3).

III. TRANSCRIPTION OF YEAST mtDNA

A. Introduction

Yeast mtDNA is larger than mammalian mtDNA and varies in complexity from 18.9 kb in *Torulopsis glabrata* to 108 kb in *Brettanomyces custerii,* with the strains of *Saccharomyces cerevisiae* — routinely used for analysis — averaging about 75 kb.[1,26] Despite the fivefold difference in size between the mtDNAs of mammals and *S.*

Table 3
METHYLATED NUCLEOTIDES IN rRNAs AND tRNAs

Organism	Species	Base methylation		Ribose methylation	
		Mitochondrial	Cell sap	Mitochondrial	Cell sap
Hamster	rRNA				
	Large	7.7	—	0.64	—
	Small	7.3	3.2	0	17.3
	tRNA	27	71	0.8	14
Yeast	rRNA				
	Large	0	1.9	0.49	10.4
	Small	0	3.0	0	9.0
	tRNA	27	46	0	5

Note: Values are expressed as methylated residues per 1000 nucleotides. Data for hamster RNA were recalculated from Dubin et al.[24] and Davenport et al.,[25] assuming a length of 1559 nucleotides for large rRNA and 953 nucleotides for small rRNA of mitochondria and a length of 2200 nucleotides for small cell sap rRNA. Data for yeast rRNAs were taken from Klootwijk et al.[50] and Martin et al.[51]

cerevisiaa, both DNAs encode basically the same set of genes (Table 1). Gene order, organization, and mode of transcription (Figure 5) are strikingly different, however, with the yeast genes scattered around the genome without obvious sense, since different orders pertain in different yeasts. The noncoding DNA that flanks genes consists of large sections of almost pure AT, sometimes interspersed with short GC-rich clusters.[27] In sharp contrast to mammalian mitochondrial genes, some yeast genes are split so that the maturation of some precursor RNAs entails RNA splicing. The genetic information is almost exclusively located in one strand of the mtDNA and there is no good evidence that extensive symmetric transcription occurs. Several sites for initiation of transcription have been found implying that at least some genes have independent promoters. This, together with the fact that most tRNAs are clustered in one quadrant of the genome,[28-30] suggests that tRNA punctuation processing is unlikely to play a major role in maturation of mRNAs.

B. Genome Organization and RNA Mapping

The sophistication of yeast mitochondrial genetics[31,32] allowed the identification of genes in mtDNA in an early stage and the availability of a physical map[33] made it possible to search for the RNAs transcribed from them. In contrast to the situation in most other organisms where rRNA genes are clustered and transcribed as a common precursor (for review see Long and Dawid[34]), the large and small rRNAs, isolated from the mitochondrial ribosome, hybridize to positions more than 28,000 bp apart on the map,[33] and this implies that coordinated synthesis of these RNAs must be achieved in a radically different way. Other RNAs have been localized to regions where genes have been identified. In some cases, however, the pattern of transcripts is complex, with several overlapping RNA species of different length occurring at positions where only one gene is expected, and their lengths far exceed that needed to code for the proteins involved.[35-37] At the same time it was found that the mtDNAs of different *S. cerevisiae* strains can vary in length as a result of the presence or absence of large insertions in the neighborhood of certain genes.[38] Both observations are explained by two related findings. First, the genes for the large rRNA,[39,40] apocytochrome *b*,[41-43] and subunit I of cytochrome *c* oxidase[44-46] are split. Second, many of the introns within these genes are optional, i.e., present in some strains but absent from others. Whether all introns are optional is as yet an open question.[47] Classification of an intron as optional depends on the finding of a strain which lacks it and so far only a limited number of strains have been looked at.

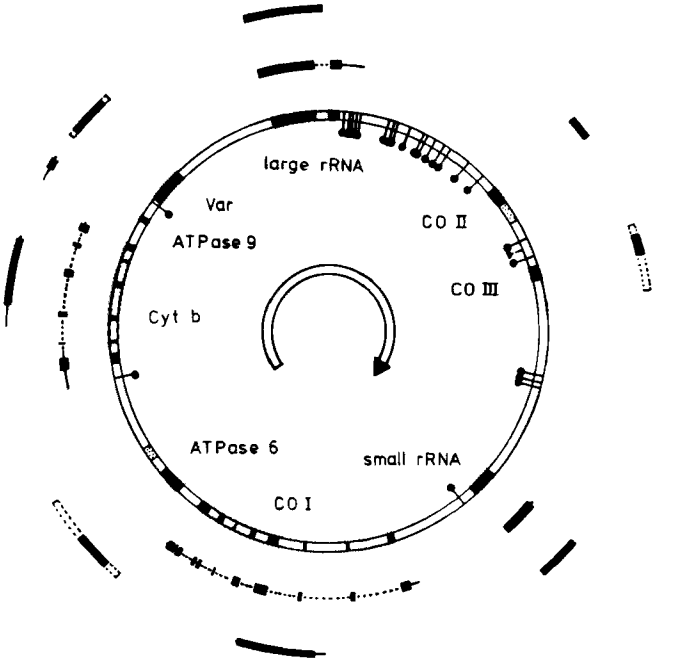


FIGURE 5. Gene organization and transcription of yeast mtDNA. The map shown is that of *S. cerevisiae* strain KL14-4A and the overall genome size is approximately 78 kb. Shaded regions identify genes or split genes, while stippled blocks outside genes indicate long reading frames with unidentified function (URF). tRNA genes are represented by small filled circles, with ∇ denoting the gene for tRNA $^{Thr}_{GAU}$, located in the opposite strand. The outer ring represents mature RNAs with possible leader and trailing sequences. Between this ring and the DNA ring precursor RNAs are indicated.

In sharp contrast to their mammalian cousins, yeast mitochondrial mRNAs have extensive 5′-leader and/or 3′-trailing sequences. For example, the mRNA from the 225-bp gene for subunit 9 of the ATPase complex is an 820-nucleotide long RNA. DNA sequence analysis, coupled with hybridization and S_1 nuclease mapping of specific restriction fragments, reveals the existence of a 500-nucleotide leader and a 70-nucleotide tail.[48,54a] The mRNA coding for apocytochrome *b* has an even longer leader of at least 940 ± 20 nucleotides and trailing sequences around 100 nucleotides. For some mRNAs, trimming of leader or trailing sequences probably occurs (see below). The 3′-termini of mRNAs have not yet been sequenced and there is no information as to whether minor posttranslational modifications can take place. However, extensive polyadenylation, as found for mammalian mtRNAs, does not occur since yeast mitochondrial mRNAs are not retained on columns of immobilized poly(U).[49]

Methylation of yeast mitochondrial rRNAs has been described (Table 3) and, as for the mammalian mtRNAs, the modifications are minimal in comparison with yeast cell-sap rRNAs, especially with regard to ribose methylation.[50,51]

C. Asymmetric Transcription

Attempts to resolve the question whether yeast mtDNA, like mammalian mtDNA, is transcribed symmetrically have led to results that have been interpreted in different ways. For example, Hendler et al.[52] observed that the amount of RNA that can be brought into hybrid with DNA is reduced after self-annealing of the RNA; they have interpreted this

as evidence for partially symmetric transcription. Further, Linnane and co-workers[53,54] have reported the isolation of a double-stranded RNA species with a complexity corresponding to the total mitochondrial genome. This RNA can only be isolated by means of a special purification protocol employing denaturing conditions. It hybridizes with a set of petite mutants representative of the whole genome and electron microscopy reveals molecules with a length up to 22 μm. This RNA species has been estimated to represent less than 0.01% of the total mtRNA, an amount which is much too low to account for the reduction in hybridization after self-annealing observed by Hendler et al.[52] and which makes further characterization difficult. In contrast to these observations are a series of experiments of different design,[1] which suggest that transcription is highly asymmetric. Thus, after a 4-min pulse with ^{32}P, only 2% of the labeled RNA was resistant to ribonuclease after self-annealing, while nascent in vitro, pulse-labeled RNA isolated from a mitochondrial transcription complex[37] or RNA pulse-labeled in isolated mitochondria was not significantly self-complementary.[55] With radioactive separated strands of restriction fragments derived from the genes coding for subunits II and III of cytochrome *c* oxidase, hybrids were found only with the coding DNA strand and no evidence was found for the existence of antimessage RNA.[56,57]

Although more experiments are obviously required to determine the significance of this low degree of symmetric transcription, there is no doubt that transcription in yeast is basically asymmetric unlike transcription in animal mitochondria.

D. Initiation of Transcription

Primary transcripts have been identified using the enzyme guanylyl transferase[58] that will cap RNAs still retaining a 5′-diphosphate terminus.[59,60] Using this technique, Christianson et al.[61] have shown that four to eight RNAs can be labeled with (α-^{32}P)GTP, indicating the existence of a number of sites for initiation of transcription. Two of these have been identified as transcripts of the rRNA genes, but the others have not been correlated yet with specific genes. Since all mitochondrial genes with the exception of that for $tRNA^{Thr}_{GAU}$[62] are encoded by one DNA strand, it will be interesting to see if one or more of the in vitro-capped RNAs will hybridize with the other DNA strand.

The nature of initiation sites has been investigated by studying the transcription of the rRNA genes. The mature 21S rRNA is capped by the enzyme guanylyl transferase indicating that the site of transcription-initiation and the 5′-end of the mature RNA coincide.[58] This contrasts with the situation for the 15S rRNA, which arises by processing at the 5′-end of a longer precursor RNA (15.5S rRNA). The 15.5S rRNA was detected in petite mutants retaining the gene for 15S rRNA, where it occurs in much higher concentration than in the wild type.[63] It overlaps with the 15S rRNA, but is approximately 80 nucleotides longer (64) at the 5′-end and it is one of the primary transcripts capped by guanylyl transferase, as mentioned above. The 15.5S rRNA is still synthesized in petite mutants retaining the 15S rRNA gene and including only 280 bp upstream of the start of RNA synthesis so that a signal for initiation of transcription is likely to be located within this segment.[64] Alignment of the 5′-ends of the two rRNAs with the DNA sequence of the genes reveals that both are preceded by identical stretches of nine nucleotides (Figure 6).[65] The same nucleotide sequence also precedes the rRNA genes of *Kluyveromyces lactis*.[57,71a] It is tempting to speculate that these regions of homology form (part of) a transcription-initiation signal on yeast mtDNA, but more work is needed to prove this.

E. RNA splicing

Whereas the regulation of initiation of yeast mitochondrial transcription is as yet poorly understood, the process of RNA splicing already has been resolved in remarkable detail. Much of our understanding is due to combined genetic and biochemical analyses

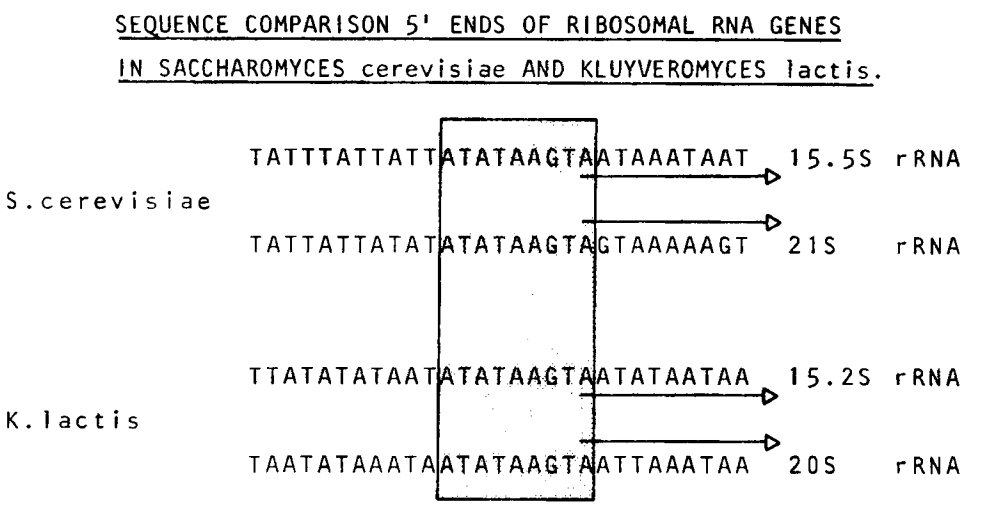


FIGURE 6. Comparison of the DNA sequences around the beginning of the rRNA genes in the mtDNAs of *S. cerevisiae* and *K. lactis*. The box indicates the region of homology and the arrows show the positions at which the rRNAs start. (From Osinga, K. A. and Tabak, H. F., *Nucl. Acids Res.*, submitted, 1982; Osinga, K. A., De Haan, M., and Tabak, H. F., *Nucl. Acids Res.*, 10, 7993, 1982. With permission.)

of the mosaic gene coding for cytochrome *b* carried out mainly by Slonimski and co-workers (reviewed by Dujon[66] and Borst and Grivell[67]). This gene can occur in various forms, dependent on strain. In so-called "long" forms, protein-coding sequences are interrupted by five introns, three of which contain long, open reading frames in phase with the preceding exons (see Figure 7). An early event in the processing of the primary transcript of the gene is the removal of the (untranslatable) first intron, thus fusing the first two exons to the reading frame contained in intron 2. This allows the synthesis of a 423-amino acid fusion protein, whose amino terminal 143 residues are contributed by cytochrome *b* exons. The fusion protein, termed the *box* 3 maturase by Lazowska et al.,[68] helps catalyze the excision of intron 2, thus destroying the mRNA which directed its synthesis. The maturase thereby regulates its own synthesis, a phenomenon which Lazowska et al.[68] call "splicing homeostasis". Mutations in the reading frame interfere with maturase function, thus, blocking processing beyond the excision of intron 1. This leads to an overproduction of defective maturase, which is detectable as a novel mitochondrial translation product, crossreactive with antibodies against cytochrome *b*.

Intron 4 also contains a reading frame continuous with exon 4, and mutations in this reading frame influence not only the splicing of the cytochrome *b* RNA itself, but also affect the maturation of cytochrome oxidase subunit I precursor RNA.[69,70] The intron 4 maturase appears to be directly involved in the excision of *OXI3* intron(s) since processing of *OXI3* precursor RNA is blocked in strains lacking intron 4 of the cytochrome *b* gene. This is an example of a mitochondrial gene regulating the expression of another mitochondrial gene.

Reading frames are also present in other introns of mitochondrial split genes and it is tempting to speculate that some of these also encode RNA maturases. As yet, however, direct evidence in support of this is lacking. In petite mutants which have retained the split gene for 21S rRNA, but which lack mitochondrial protein synthesis, faithful splicing of the 21S rRNA precusor still takes place.[71] It is, thus, unlikely that the protein specified by the URF in the intron of this gene is required for RNA splicing and this must, therefore, be carried out by an imported, nuclear-encoded enzyme. Some introns of the cytochrome *b* and *OXI3* gene are also excised in petite mutants lacking mitochondrial

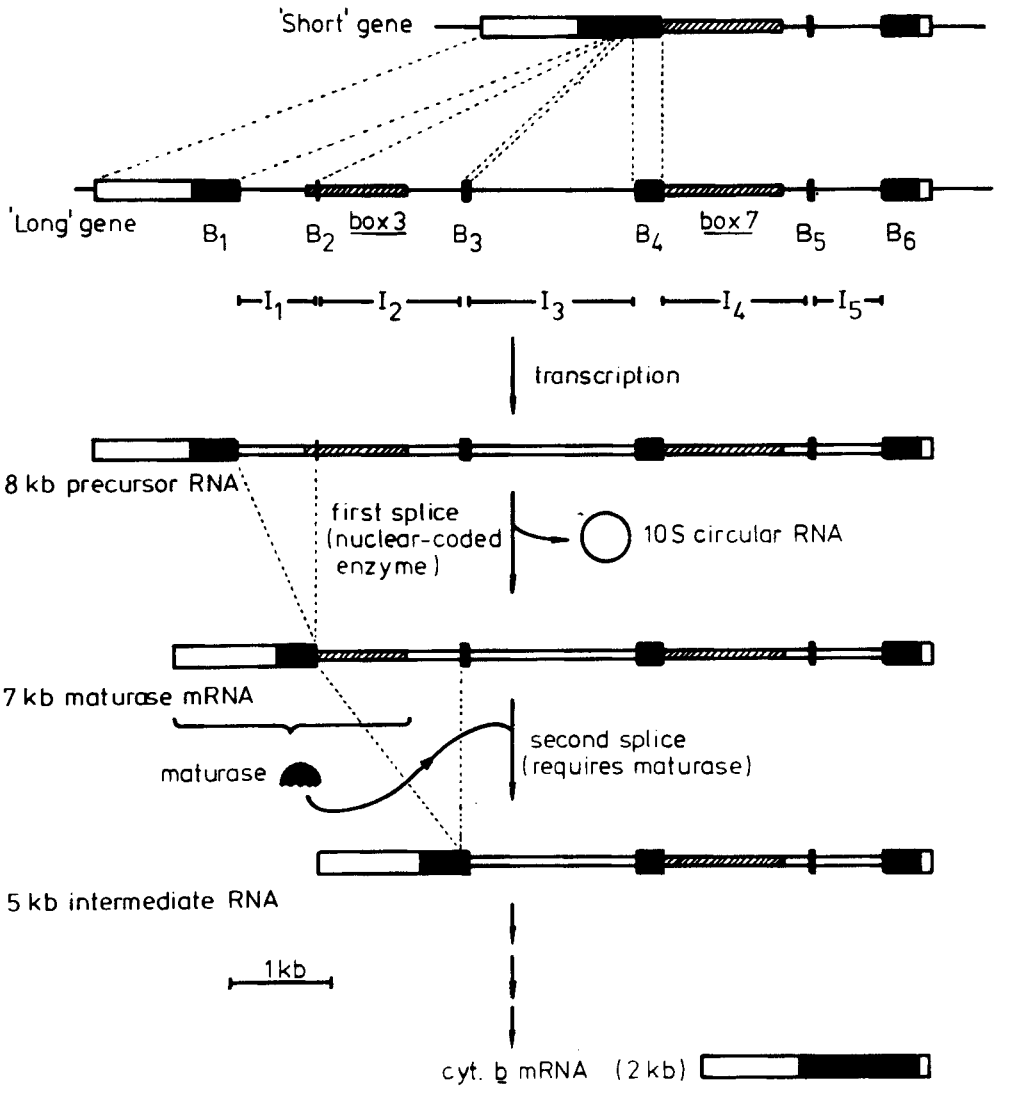


FIGURE 7. The structure of the "long" and "short" versions of the gene for apocytochrome *b* and of the mRNAs transcribed from the "long" gene. Black boxes correspond to the exons E1-E6 which specify the amino acid sequence of apocytochrome *b*; stippled blocks designate the nontranslated regions of the mRNA for cytochrome *b*; hatched blocks represent long open reading frames in the introns of the *b* gene. Whether the 8-kb precursor RNA is the primary transcript or not is uncertain. See text for further explanation.

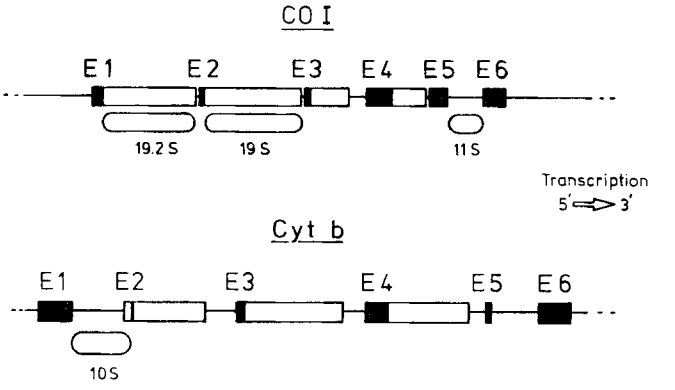
protein synthesis (Table 4). This shows that splicing of mitochondrial RNAs requires an intricate interplay between nuclear and mitochondrially specified functions.

Mutations affecting maturase structure or function are not the only ones which disturb certain mitochondrial splicing events. In addition to this class of mutants (generally recognizable by the fact that they are complementable in *trans*), several groups have reported the isolation of *cis*-dominant splicing-defective mutants. These mutations are located not only at exon-intron borders, but also at sites within introns, and presumably they result in defective recognition sites for splicing enzymes. This offers unique possibilities to identify RNA sequences essential for intron excision.

An interesting discovery made during electron microscopic analysis of mtRNA preparations was the occurrence of RNA circles. These are resistant to several drastically

Table 4
PROPERTIES OF CIRCULAR RNAs FROM YEAST MITOCHONDRIA

Circle	Gene	Excised in petites	Open reading frame
11S	COI	+	–
19.2S	COI	–	+
19S	COI	–	+
10S	Cyt. *b*	+	–



Note: COI is the gene for subunit AI of cytochrome *c* oxidase as found in *S. cerevisiae* strain D273-10B; cytochrome *b* is the gene for apocytochrome *b* as found in *S. cerevisiae*, strain KL14-4A.

denaturing treatments, which strongly suggests that they are covalently closed. They correspond exactly with the presence of introns (Table 4) (see Arnberg et al.[72] and Halbreich et al.[73]) and a plausible suggestion is that they are by-products of splicing. Their relative abundance in mtRNA preparations could mean that they are more than just fleeting intermediates in RNA processing.

F. Other Forms of RNA Processing

The synthesis of 21S rRNA has been studied in wild-type cells[74] and petite mutants,[71,75] and precursor RNAs have been found with a long 3′ extension of approximately 1 kb[75] (see Figure 5). A preference for priority in trimming or splicing was not observed. In contrast to the 21S rRNA, the 15S rRNA is derived by trimming an RNA with an 80-nucleotide extension at the 5′-end of the RNA.[64] End-group analysis of 15S rRNA shows heterogeneity at the 5′-end which could be an indication for imprecise processing at any of five adjacent nucleotides.[61]

Evidence for trimming also has been obtained with precursors of two mRNAs. The gene coding for subunit II of cytochrome *c* oxidase lacks intervening sequences, yet restriction fragments containing internal sequences of this gene hybridize to several overlapping transcripts. The largest of these RNAs is more than 3000 nucleotides and appears to be processed in several discrete steps into the mRNA of 850 nucleotides.[57] Since the gene is uninterrupted, this implies that trimming at ends is responsible for mRNA maturation in this case, if the longer transcripts are indeed precursor RNAs. Transcripts of different length were also found in the region of mtDNA where the uninterrupted gene coding for subunit 6 of the ATPase complex is located;[36] trimming of precursor RNA into mRNA may also occur in this case.

Even with the existence of exceptionally long extragenic RNA segments there are large stretches of mtDNA left which do not hybridize detectably with RNA. This suggests that

RNAs arise by recognition of proper DNA-encoded start and stop signals for mtRNA polymerase rather than by processing of multicistronic RNAs. The tRNAs may be an exception to this, however. During studies on transcription in petite mutants, Levens et al.[76] observed that the appearance of mature tRNAs was dependent on the presence of a region of the mitochondrial genome located between the small rRNA gene and the gene coding for subunit III of cytochrome *c* oxidase and they, therefore, proposed that a mitochondrial function might be involved in tRNA processing. This has been analyzed in more detail by Martin and Underbrink-Lyon.[77] Petite mutants retaining the gene for $tRNA^{Ser}$ lack mature tRNA, but contain longer RNAs that hybridize with a specific probe for this tRNA. Complementation of these mutants by mating with petites retaining the "tRNA synthesis" locus — a mtDNA region located between the *OXI2* locus and the 15S rRNA gene — leads to appearance of normal $tRNA^{Ser}$ even though mating does not lead to restoration of mitochondrial protein synthesis. This suggests that the factor supplied by the tRNA synthesis locus is an RNA and that it acts at the level of transcription or posttranscriptional processing.

IV. IN VITRO TRANSCRIPTION OF mtDNA

A. RNA Synthesis in Isolated Mitochondria

Although complete reconstitution of mitochondrial transcription in vitro from purified components is a cherished goal for many a biochemist, it is not an easy one to attain, so that several attempts have been made to study RNA synthesis in isolated mitochondria in vitro.[55,78,79] It has been found that (^{32}P)UTP is linearly incorporated for more than 1 hr into RNA of discrete length and that this newly synthesized RNA hybridizes with mtDNA. The labeled RNA is inaccessible to added ribonuclease and incorporation is not dependent on added Mg^{2+}, which strongly suggests that synthesis takes place inside intact mitochondria. UTP incorporation is inhibited by ethidium bromide and acriflavin, but is not affected by the presence of chloramphenicol, suggesting that RNA synthesis is not tightly linked to mitochondrial protein synthesis. Some of the RNA products display labeling kinetics characteristic of precursors and the hybridization behavior of two of these closely resembles that expected for bona fide precursors to the rRNAs. In the case of the large rRNA, the precursor appears to undergo complete processing, including trimming at the 3'-end and splicing during a chase. For the small rRNA, however, the 15.5S precursor RNA could not be chased into mature 15S rRNA.[78]

While isolated mitochondria permit the labeling of RNAs to high specific activity, making them amenable to detailed structural analysis, their use for analysis of transcription has intrinsic difficulties. First, the specific activity of the RNAs synthesized can vary enormously as a result of dilution with unlabeled RNA already present in the mitochondria. Thus, interpretation of hybridization patterns is complicated by variable and unpredictable competition from unlabeled RNA species. Further, it is not always easy to prove that RNAs are bona fide intermediates in processing and not due to degradation occurring in mitochondria taken out of their natural environment. Finally, a shortage of essential factors, normally supplied from the cell sap, can prohibit further maturation (see results for 15.5S rRNA), and it may turn out to be difficult to supply such factors in vitro because of the impermeability of the inner mitochondrial membrane.

B. mtRNA Polymerase

Several attempts have been made in the past to purify mtRNA polymerase, but not in all cases have the problems of contamination by nuclear RNA polymerases been overcome. mtRNA polymerase has been purified from *Neurospora* and the properties reported make it an attractive candidate for an authentic mitochondrial enzyme.[80] It

prefers its own mtDNA template and is sensitive to rifampicin. However, this work has seen no follow-up. mtRNA polymerase has also been purified and characterized from *Xenopus* oocyte mitochondria, but the enzyme has not been used for in vitro reconstitution experiments.[81]

Of all mitochondrial RNA polymerases, that from yeast mitochondria is, at the moment, the best characterized thanks to the work of Levens et al.[82] The most highly purified preparations of the enzyme sediment at 6.3S in glycerol gradients commensurate with a molecular weight of 90 kD, assuming a globular structure. It is probably a dimer, since SDS gel electrophoresis of active fractions reveals a single, major polypeptide of 45 kD. Antibody raised against this polypeptide inhibits activity of both the purified enzyme and the transcription complex in which the enzyme is still bound to its original template. Since the transcription complex preferentially synthesizes mitochondrial rRNAs,[83] this is strong support for the view that this RNA polymerase is an authentic mitochondrial enzyme. Other properties confirm this. In contrast to the nuclear polymerases, the mitochondrial enzyme is inhibited by Mn^{2+} and elevated ionic strength, it is rather resistant to rifampicin, and mtDNA is the most efficiently transcribed natural DNA next to the artificial polymer poly(dAT). In recent reconstitution experiments, successful initiation of transcription has been shown to occur. Restriction DNA fragments containing the 5′-half of the rRNA genes with flanking sequences direct the synthesis of specific run-off transcripts with lengths dependent on the position of the restriction site used for artificial termination of transcription.[87a]

Since mtRNA polymerase plays a central role in mtDNA expression, regulation of the synthesis of RNA polymerase could be one of the ways in which the nucleus exerts control over mitochondria. The biosynthesis of mtRNA polymerase has been studied by translation of RNA, derived from yeast cells in different growth stages, in a reticulocyte lysate. This gives rise to a protein of 47 kD that is immunologically related to the 45-kD subunit of the polymerase and probably is the precursor of RNA polymerase synthesized in the cell sap. RNA isolated from cells harvested in late-logarithmic or stationary phase, directs the synthesis of increased levels of the 47-kD protein. This increase follows the same time course as the induction of the cytochromes and thus can be correlated with the increase of synthesis of proteins destined for mitochondrial biogenesis.[84] It remains to be seen, however, whether mtRNA polymerase plays a primary role in this circuit of regulatory processes.

V. ASPECTS OF MITOCHONDRIAL TRANSCRIPTION IN OTHER ORGANISMS

The concentration of research efforts on the structure and function of human and yeast mtDNAs has yielded reasonably detailed pictures of transcription and RNA processing. In other organisms, information is still limited to a somewhat sketchy knowledge of gene organization. In some instances, however, this organization is such that a number of interesting questions with respect to transcription arise. These are discussed briefly below.

A. Transcription of Other Animal mtDNAs

The complete DNA sequence of human mtDNA recently has been complemented with DNA sequences of mouse,[6] cow,[3] and rat[85] mitochondria. These mtDNAs display minor differences, many of which are clustered in a 1500-bp nontranscribed region containing the H-strand origin of replication (D-loop region). Overall genome organization is conserved, however, indicating that the principles of transcription and processing laid down for human mtDNA probably also hold for other animal mtDNAs. So far, HeLa is the only cell type in which detection of L-strand transcripts has proved possible, despite

efforts with other cells and organisms.[86] This is probably a technical problem, since the L-strand has, in all cases, a limited coding function. We expect that symmetric transcription is a typical trait of animal mitochondria.

B. Transcription of mtDNA in *Ascomycetes*

Gene organization in the *Ascomycetes* is in outline, at least, similar to that of yeast: the mtDNAs are rather large and split genes have been found.[87-91] In *Neurospora,* the gene for the large mitochondrial rRNA is split and the intron within it is twice the size of the corresponding intron in yeast. Nevertheless, it is located at the same position within the gene and it also contains a reading frame that can code for a protein 258 amino acids long.[92]

In *Neurospora,* nuclear temperature-sensitive mutants, disturbed in the processing of large ribosomal precursor RNA (35S RNA), have been found.[93] The 35S RNA still contains the 2300-nucleotide intron suggesting that the mutations are located in nuclear genes whose proteins are involved in the mitochondrial splicing process. The RNA can be isolated from the mutants as a ribonucleoprotein particle associated with almost the full complement of large subunit ribosomal proteins. A hairpin structure, located in the intron sequence near the 5′ exon boundary, is susceptible to ribonuclease III of *E. coli* in such particles, suggesting that the intron in a partially assembled ribosomal particle may be accessible to splicing enzyme(s). This particle may, therefore, be the natural substrate for RNA splicing. From a practical standpoint these mutants are an excellent source of precursor RNA that can be used as a substrate in the characterization of a splicing enzyme.

Studies on gene localization have shown that the rRNA genes and most of the tRNAs are found on a DNA segment of about 20 kb, all encoded on the same DNA strand, implying that transcription is asymmetric. Transcription runs from the small to the large rRNA gene and analysis of RNA blots with radioactively labeled restriction fragments shows the presence of long RNAs for this rRNA-tRNA region. The authors suggest that mature rRNAs and tRNAs probably arise by processing of longer transcripts.[94] In this connection it may be significant that the ribosomal genes and most (groups of) tRNA genes are flanked by inverted repeats consisting of two PstI sites separated by a TA dinucleotide.[92] These hairpin structures in precursor RNA could function as recognition signals for an enzyme with properties comparable to *E. coli* ribonuclease III. This could cut the precursor RNA into smaller units in preparation for final trimming leading to mature rRNA and tRNAs.

In summary, these results indicate that nature has not selected out one favored type of mtDNA within the fungi and this is a situation which contrasts sharply with that encountered in the animal mtDNAs.

C. Transcription of Plant mtDNA

Transcription of plant mtDNA is an essentially unexplored field, due mainly to an almost complete ignorance of gene complement and structure. The total complexity of mtDNA in various plants has been estimated to exceed 120 kb, and this high complexity also is reflected in the larger number of mitochondrial translation products seen on SDS-acrylamide gels compared with mitochondria of other organisms.[95] Virtually all of these translation products await further characterization, however.[96]

By use of the yeast mtDNA fragment containing the gene coding for subunit II of cytochrome oxidase as a specific hybridization probe, Fox and Leaver[97] have succeeded in isolating the gene coding for the same subunit in maize mitochondria. DNA sequence analysis reveals the existence of a 794-bp-long intervening sequence and RNA blot hybridizations, with specific exon or intron probes, have permitted transcription to be studied. At least three precursor RNA species retaining the intron can be detected and

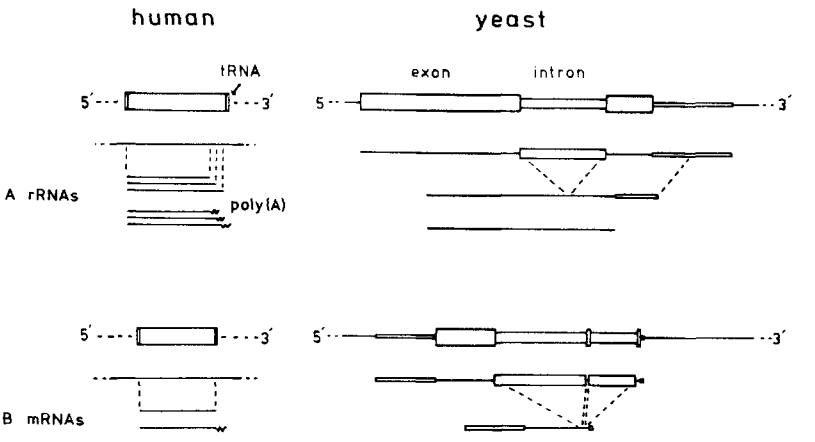


FIGURE 8. Transcription of mitochondrial genes in man and yeast. The figure compares and contrasts the mode of expression of the genes for the large rRNA and for apocytochrome *b*. For the latter yeast gene, the structure shown is that of a so-called "short" version.

several abundant RNAs are found to hybridize with the exon probes. The lengths of these exceed that of the coding sequence. The transcription pattern is surprisingly complex when compared with the transcription of the same mitochondrial gene in yeast.

D. Transcription of mtDNA in Trypanosomes

Perhaps the most bizarre form of mtDNA is found in the mitochondria of trypanosomes (for review see Englund et al.[98] and Borst and Hoeijmakers[99]). The enormous amount of mtDNA can be visualized in the light-microscope by histochemical staining procedures for DNA. It consists of small, interlocked DNA circles forming a complex network. These circles are heterogeneous in sequence and display a high rate of sequence evolution. They are not transcribed. Larger circles (maxicircles) can be seen extending from the DNA network and these do hybridize with RNA and are considered to be the functional counterpart of mtDNA, observed in other organisms. The minor maxicircle transcripts bind to oligo(dT)-cellulose and probably represent polyadenylated mitochondrial mRNAs. The rRNAs are very small (about 640 and 1230 nucleotides) and have only weak homology with rRNAs from any other source, including animal mitochondria.[100] The genes for these RNAs are adjacent, but transcribed in the direction 5'-large-small-3', i.e., opposite to the usual direction found in bacteria, chloroplasts, or animal mitochondria.[101] With their bizarre mtDNA and mitochondrial rRNAs, trypanosomes may have other surprises in store for those analyzing mitochondrial transcription in more detail.

VII. SUMMARY

A striking aspect of mitochondrial transcription is the lack of uniformity in which different organisms express their mitochondrial genomes (Figure 8). Animal mitochondria have reduced the number of transcriptional starts to the minimum: both DNA strands are completely transcribed from single initiation points on each DNA strand and gene-specific RNAs are generated by extremely precise cleavage of the primary transcripts. The secondary structures of tRNA sequences within these transcripts are thought to play an essential role in this process (tRNA punctuation model). Differential control of gene expression may be realized by transcription-attenuation, control on the

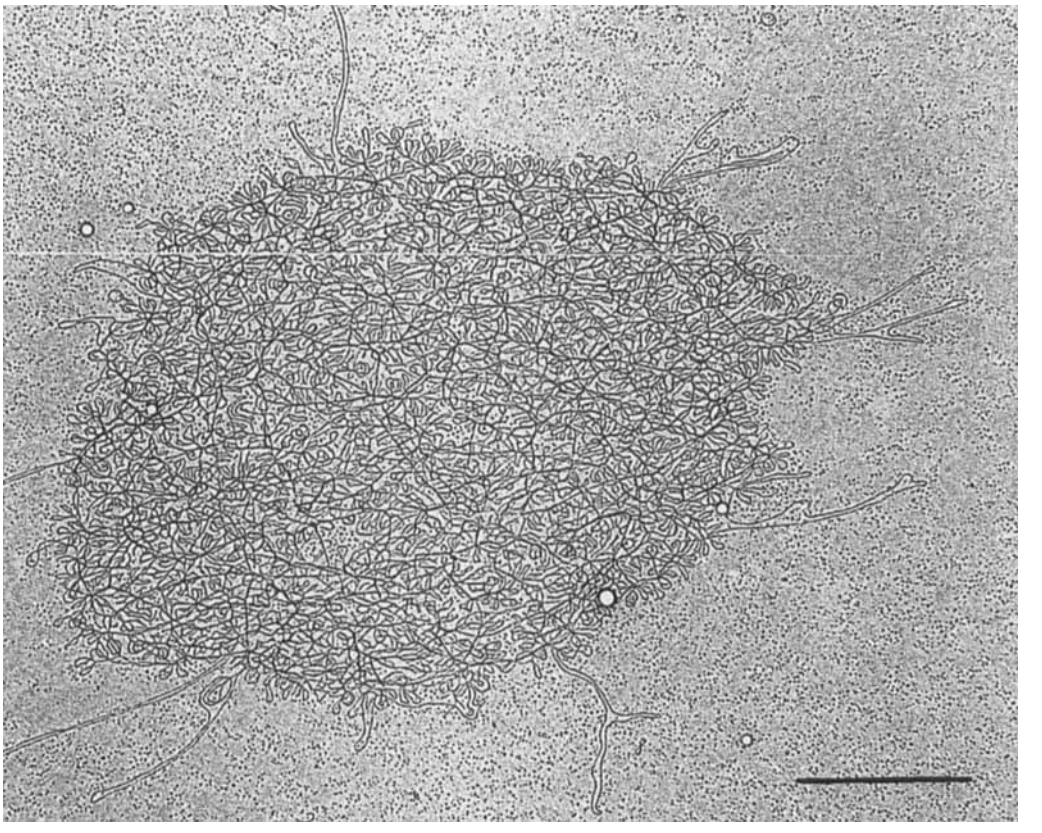


FIGURE 9. Electronmicrograph of kinetoplast DNA of *Trypanosoma brucei*. This large network consists of many interlocked minicircle DNAs and some maxicircle DNAs that can be seen extending from the edge of the complex. The bar corresponds to 1 μm.

cleavages required to generate mature RNAs, and by the differences in stability of these RNAs.

Transcription of yeast mtDNA conforms to principles more generally found in nature, in that expression of single genes or groups of genes is regulated by recognition of individual promoter sites. The fact that purified yeast mtRNA polymerase initiates transcription correctly on DNA restriction fragments in vitro promises to be a breakthrough in the study of mitochondrial transcription. The presence of split genes in this DNA demands the existence of a cut-and-splice mechanism. Both nuclear and mitochondrial genes contribute to this, since some splicing events are mediated by proteins, specified wholly or in part by intron sequences. Certain splicing steps give rise to novel products in the form of covalently-closed circular RNAs. Whether these RNAs fulfill an independent function or whether they are merely by-products of splicing has yet to be resolved.

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