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Detection, Localization, and Sequence Analyses of Mitochondrial Regulatory Region RNAs in Several Mammalian Species[1]

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The mitochondrial regulatory region (mrr) located between the $tRNA^{Phe}$ and $tRNA^{Pro}$ genes of mitochondrial DNA (mtDNA) is essential for regulation of replication and transcription of the mitochondrial genome. Polyadenylated short RNAs complementary to the L-strand of the mrr in human cells and similar RNAs (polyadenylation status unknown) in rat and mouse cells have been reported. We now report detection of *ca.* 0.2 kb polyadenylated mrrRNAs in cultured cells of Chinese hamster, African green monkey, mouse, rat, and human. We isolated a cDNA clone to a rat polyadenylated mrrRNA of 158 bp in length excluding the polyadenyl tail, which spans the region from the light strand promoter (LSP) to the origin of heavy strand replication (OriH). This cDNA contains both an open reading frame encoding a 26 amino acid polypeptide and a 12 nucleotide sequence complementary to the 3′-terminus of rat mitochondrial 12S rRNA. A cDNA clone to a human HeLa cell polyadenylated mrrRNA also contains a 12 nucleotide region complementary to the human mitochondrial 12S rRNA. We used a mitochondrial genome-deficient HeLa cell line, ρ^0 HeLa, and a derived cybrid cell line, HeEB, with a reconstituted mitochondrial genome, to demonstrate that the occurrence of the mrrRNA is dependent on the presence of a mitochondrial genome, and these polyadenylated mrrRNAs are transcribed from the mitochondrial genome. Our results further substantiate the common existence of polyadenylated mrrRNAs among mammals and support previously proposed hypotheses for the multi-functional nature of polyadenylated mrrRNA.

Key words: cDNA, mitochondrial DNA, mitochondrial RNA, mitochondrial transcription, polyadenylated RNA.

The mammalian mitochondrial DNA (mtDNA) consists of a *ca.* 16 kbp closed circular double-stranded molecule (*1-3*). The mitochondrial regulatory region (mrr) is located between $tRNA^{Phe}$ and $tRNA^{Pro}$ sequences and is essential for mtDNA replication and mitochondrial RNA (mtRNA) transcription. The nucleotide sequence of the mrrDNA is highly variable among species, yet several common structural features are conserved, such as light and heavy strand promoters (LSP, HSP), a displacement-loop (D-loop) region, an origin of heavy strand replication (OriH), and conserved sequence blocks (CSBs) (*4-11*).

RNAs of *ca.* 0.2 kb of heterogeneous size with sequences complementary to the L-strand of mrrDNA have been described in human (*7, 12, 13*), mouse (*14*), and rat (*15*). Typically, these RNAs have 5′ ends which approximate the LSP and their 3′ ends within CSBs or near OriH. Some of these RNAs in human cells have been shown to be polyadenylated (*12, 13*). RNA species with their 3′ ends covalently attached to or coincident with the 5′-end of H-strand DNA sequence have also been reported in mouse (*14*) and in human (*7*). We will refer to these various RNA species as mrrRNA for mitochondrial regulatory region RNA.

Owing to the great variation in mrrDNA sequences among species (*9, 10, 15-17*), there is a paucity of data concerning the extent to which mrrRNAs occur in other mammals. African green monkey mrrDNA contains a unique 108 bp triple repeat which could not only confound detection of mrrRNAs but also complicates predictions as

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Abbreviations: bp, base pair; CSB, conserved sequence block; ctRNA, cytoplasmic RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP, heavy strand promoter; LSP, light strand promoter; mrrDNA, mitochondrial regulatory region DNA; mrrRNA, mitochondrial regulatory region RNA; mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; rRNA, ribosomal RNA; $tRNA^{Phe}$ and $tRNA^{Pro}$, transfer RNAs for phenylalanine and proline; OriH, origin of heavy strand replication; poly(A^+) RNA, polyadenylated RNA; ρ^0 HeLa, mitochondrial genome-deficient HeLa cell line.

to the likely function of these mrrRNAs if they exist in this species (*17*).

In human, the mrrRNAs have been called DHP-RNA (*7*), 7S RNA (*12*), or RNA 18 (*18*). The 7S RNA was purified from the mitochondrial fraction of HeLa cells and sequenced at its 5′-end proximal segment, while the site of 3′-polyadenylation was not determined precisely (*12*). cDNA clones were obtained to polyadenylated mrrRNAs from human diploid fibroblast cell line, IMR-90, and designated as IB43 and IB65 (*13*). Even though these cDNA sequences overlap, they contain polyadenyl tails at distinct positions, and the 5′ end of IB43 maps to a site 40 nucleotides downstream to that determined for 7S RNA. Thus it is not clear whether termini of mrrRNAs are generally heterogeneous or even whether longer mrr cDNAs might be obtained.

We reported previously that mrrRNA production is chemically inducible in normal human fibroblasts, but not in the fibroblasts from patients with an autosomal recessive disorder, and suggested the possibility that mrrRNA production is dependent on a nuclear mrrDNA-like sequence (*13, 19*). Recently others isolated a human genomic DNA clone which contains a mitochondrial D-loop sequence (*20*). Though this clone lacks the region between LSP and oriH, this is consistent with the presence of potential templates for mrrRNAs in human chromosomal DNA. The presence of mrrDNA-related sequences in chromosomal DNA has been reported in other species (*21-25*). Definitive experiments using mitochondrial genome-deficient cell lines and mitochondrial genome-restored cybrid cell lines (*26-28*) could clarify the location and origin of mrrRNAs; specifically, whether they are transcribed from nuclear or mitochondrial DNA.

We have examined several mammalian species for the presence of polyadenylated mrrRNAs. We found that African green monkey (CV-1), Chinese hamster ovary (CHO), human (HeLa), mouse (BALB/3T3), and rat (JTC-19) cells all contain mrrRNAs complementary to the L strand. These mrrRNAs are heterogeneous in size, particularly in the African green monkey cells. We used RT-PCR and cloned cDNAs to mrrRNAs to map the termini and determine the sequences contained in representative human and rat mrrRNAs. We report the first CHO cell sequences for the mrrDNA including the conserved sequence blocks. Furthermore, we used the mitochondrial genome-deficient cell line ρ^0 HeLa, and the HeEB cybrid cell line derived from ρ^0 HeLa, to demonstrate that mrrRNAs are transcribed from the mitochondrial genome in human cells.

MATERIALS AND METHODS

Cell Lines—For cell culture, BALB/3T3 mouse fibroblast cell line, CV-1 African green monkey kidney cell line, HeLa S3 human cervical carcinoma cell line, JTC-19 rat lung fibroblast cell line, and CHO K-1 Chinese hamster ovary cell line were grown in Eagle's MEM (GIBCO BRL, Grand Island, NY), supplemented with non-essential amino acids and 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS). Mitochondrial genome-deficient ρ^0 HeLa cells and cybrid cells, HeEB (a reconstituted cell line obtained by fusing ρ^0 HeLa with enucleated HeLa cells) were grown in glucose-rich RPMI 1640 medium supplemented with 0.1 mg/ml pyruvate and 10% v/v FBS (*26-28*).

RNA Extractions—Total RNAs were obtained from the cultured cells by homogenization in guanidium isothiocyanate/phenol (*29*; RNAzol B, Biotex Laboratories, Houston, Texas). Poly(A^+) RNAs were selected from total RNA by chromatography on Oligotex-dT30<super> (Daiichi Pure Chemicals, Tokyo) according to the manufacturer's protocol.

RT-PCR and PCR—Templates for the reverse transcription-polymerase chain reaction (RT-PCR) were first-strand cDNAs generated from one microgram of poly(A^+) RNA using the Ready-to-Go T-primed First-strand Kit (Pharmacia Bio Tech, Tokyo). Rat mrr cDNAs were separately amplified using the L-strand 5′ primer sequences RP1 (5′TTTGTGGTCTACTAGAATGG3′) and RP2 (5′ GAAGCCTTTTAAGAATTAAG3′) (*2*; Genbank X14148). PCR of the first-strand cDNA was carried out using either of the 5′-primers and an oligo d(T) 3′-primer, and AmpliTaq DNA polymerase (Perkin Elmer, Chiba) according to the manufacturer's protocol except that the annealing temperature was 50°C instead of 55°C. Human mrr cDNAs were amplified as above except that primer sequences used were P1 (5′TTTGTGGTCTACTAGAATGG3′) and P2 (5′ GAAGCCTTTTAAGAATTAAG3′). RT-PCR amplified DNAs were subsequently cloned into a pUC19 plasmid vector for nucleotide sequence determination (Takara Shuzo, Shiga).

Probe DNAs—Species- and strand-specific oligonucleotide probes were based on published sequences for the mitochondrial L-strand and synthesized using an Applied Biosystems DNA synthesizer (Perkin Elmer): human specific probe, 5′TTCCACACAGACATCATAACAAAAA-ATTTCCACCAAACCC3′ (*1*, Genbank J01415); African green monkey specific probe, 5′ACACTTAAAAAAAATT-CAAAAAAAATCTTTAATCAAACCC3′ (*17*, Genbank X05609); rat specific probe, 5′ATAAATATTTATAAATA-CTGAAAACTCTGTCAACAAACCC3′ (*2*, Genbank X14148); mouse specific probe, 5′ATAAATGCTACT-CAATACCAAATTTTAACTCTCCAAACCC3′ (*3*, Genbank J01420); and CHO specific probe, 5′cGACATATCTT-TAATTTACACTAGATTTTAAgCCAAACCC3′ (lower case letters c and g were corrected to G and C respectively, which appear in the DDBJ D29972). For use as hybridization probes, oligonucleotides were end-labeled using [γ-^{32}P]ATP (7,000 Ci/mmol, ICN Biochemicals, Costa Mesa, CA) and T4 polynucleotide kinase (Takara). Other hybridization probes included a pUC19 plasmid containing a 550 bp *Hin*dIII/*Xba*I fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA excised from pHcGAP (*30*, ATCC 57090), and pUCCoxIII containing a PCR amplified portion of the human cytochrome oxidase III gene from HeLa S3 cell DNA, the identity of which was confirmed by partial nucleotide sequencing. A plasmid clone of a IB43 cDNA, pUCIB43, was kindly provided by Dr. A. Noda (*13*). Plasmid probes were labeled using [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham) and a Random Primer DNA Labeling kit (Takara).

Northern Analysis—For Northern blot hybridization, RNA samples were electrophoresed in 1.5% agarose gels containing formaldehyde, transferred to Hybond N^+ nylon membrane (Amersham), and hybridized with radioactively labeled probes.

DNA Sequencing and Computer Analyses—RT-PCR-amplified cDNAs were cloned into pUC19, and sequences were determined for both strands by the dideoxy chain termination method (Sequenase Ver. 2, United States Biochemical, Cleveland, OH; Sequencing Pro kit, TOYOBO, Tokyo or ABI automated DNA sequencer, Perkin Elmer) using M13 universal or reverse primer. Similarly the mrrDNA amplified from CHO was cloned into pUC19, and sequences were determined as above for three independent plasmid clones. ORF analyses were performed using the ORF search program (Genetyx Software Development, Tokyo).

RESULTS

Identification of CSBs in the mrrDNA of CHO Cells—Because the sequence information of mrrDNA for CHO cell was not available, we first amplified mrrDNAs using the degenerate primers (see legend to Fig. 1) and CHO cell total genomic DNAs. Mouse total genomic DNA was used as a reference to assess authenticity of degenerate primers. The primers directed a single amplification product of *ca.* 900 bp in agreement with the expected size for mouse, and a product of *ca.* 920 bp from CHO DNA (data not shown). Sequence results revealed that the CHO mrrDNA consisted of 878 nucleotides located between the 5′ ends of the mtDNA $tRNA^{Phe}$ gene encoded by the H-strand and the $tRNA^{Pro}$ gene encoded by the L-strand. Percent identities for the aligned sequences of mrrDNA are 29.7% between Chinese hamster and rat, 31.4% between Chinese hamster and mouse, and 44.8% between mouse and rat. The nucleotide sequence data of CHO mrrDNA used in this paper will appear in the DDBJ nucleotide sequence database with the accession number D29972. We used CHO mrrDNA nucleotide sequence to determine CSBs for this species by comparison with the previously determined CSBs for rat and mouse (*4*, Fig. 1).

Detection of mrrRNAs in the Cultured Cells of Five Mammalian Species—First, we PCR-amplified double-stranded mrrDNA segments containing CSBs and the LSP from Chinese hamster, African green monkey, human, rat, and mouse DNAs using an internal consensus 5′ end primer (5′CGTTCCCCTTAAATAAGAC3′) with the $tRNA^{Phe}$ gene degenerate sequence as 3′ end primer (shown in legend to Fig. 1), and used them as probes (data not shown). These amplified segments detected a *ca.* 0.2 kb band in each of the total RNAs isolated from the five species (Fig. 2A). An additional band at *ca.* 0.28 kb was clearly detected in the African green monkey RNA (Fig. 2A, lane b). Multiple bands ranging from *ca.* 0.28 kb to *ca.* 3 kb were detected in the three rodents, also indicating the presence of multiple overlapping transcripts (Fig. 2A, lanes c, d, and e). Next, to see the strand specificity and polyadenylation status, we synthesized oligonucleotide probes with an L-strand sequence for the region between CSB1 and CSB3 in each species, and prepared polyadenylated RNAs. These

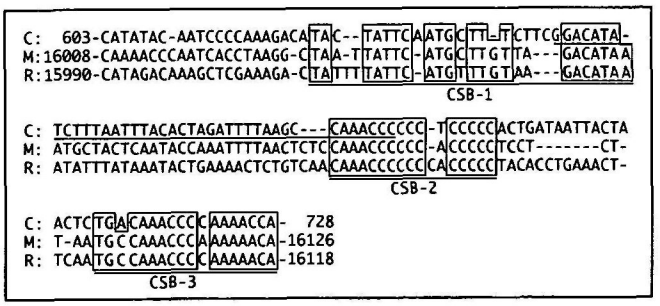

Fig. 1. **Identification of conserved sequence blocks (CSB) in the CHO mrrDNA.** Degenerate PCR primers flanking the mrr were synthesized according to aligned sequences for $tRNA^{Phe}$ and $tRNA^{Pro}$ genes in the mtDNA of rat, *Rattus norvegicus* JTC-19 cell (*2*), mouse L cell (*3*), and human placenta/HeLa cell (*1*). Primer sequences are $tRNA^{Pro}$ gene 5′CACCAYYARCACCCAAAGC3′ (Y: C or T, R: A or G) and $tRNA^{Phe}$ gene 5′GKNRKTAAGCTACRTWRAC 3′ (K: G or T, W: A or T, N: A or G or T or C). These primers were used for PCR amplification of mrrDNA from purified total genomic DNA of Chinese hamster CHO cells. The nucleotide sequence for the mrrDNA of Chinese hamster (C) was determined (DDBJ accession number: D29972) and its sequence was compared with published sequences for mouse (M) (EMBL accession number: J01420) and rat (R) (EMBL accession number: X14848) to identify the CSBs. Boxed sequences for CSBs in mouse and rat were used as references according to the previous report (*4*) in order to identify CSBs in the CHO sequence. Gaps ('-') were inserted to maintain alignment. Underlined sequence in the CHO (nucleotide numbers 644–685) was used as a species- and strand-specific probe for Northern blot analysis.

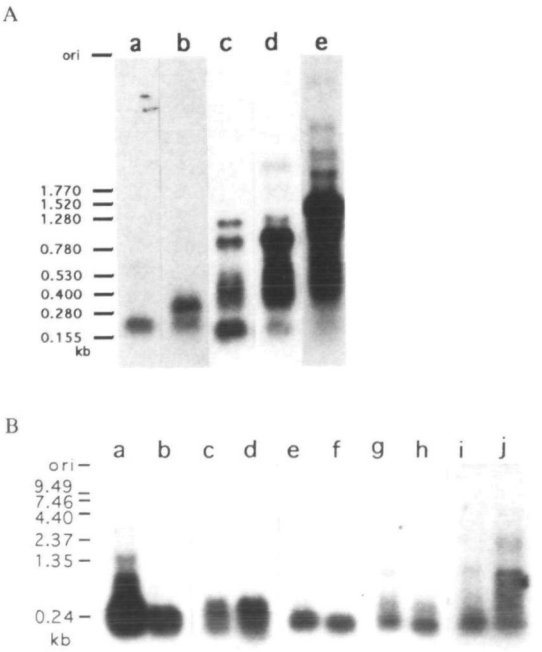

Fig. 2. **Detection of *ca.* 0.2 kb mrrRNAs in five mammalian species by Northern blot analyses.** (A) Total RNAs were electrophoresed, transferred by Northern blotting and hybridized with ^{32}P-labeled mrrDNA segments amplified by PCR for each species. RNAs analyzed were from human (lane a), African green monkey CV-1 (lane b), rat (lane c), mouse (lane d), and Chinese hamster (lane e). Each lane was loaded with 10 μg of total RNAs. Size markers indicated on the left are a 0.16–1.77 kb RNA ladder (Gibco BRL). (B) Total and poly(A^+) RNAs were electrophoresed, transferred by Northern blotting and hybridized with ^{32}P-labeled species-specific oligonucleotide probes described in "MATERIALS AND METHODS." RNAs analyzed were from human (lanes a and b), African green monkey (lanes c and d), rat (lanes e and f), mouse (lanes g and h), and Chinese hamster (lanes i and j). Lanes a, c, e, g, and i were loaded with 10 μg of total RNAs while lanes b, d, f, h, and j were loaded 5 μg of poly(A^+) RNA. Size markers indicated on the left hand side are from a 0.24–9.5 kb RNA ladder (Gibco BRL).

species- and strand-specific probes each detected a *ca.* 0.2 kb RNA band in their respective species total RNA. Other RNA bands ranging from 0.3 to 1.3 kb were also detected in the total RNA, but they were drastically reduced in rat, mouse and Chinese hamster (Fig. 2B, lanes e, g, and i, compare with Fig. 2A). The polyadenylated RNAs showed further reduced extra bands particularly in human and mouse (compare Fig. 2B, lanes a and b, or g and h). The rat and human polyadenylated RNAs produced only a single observable RNA band, respectively (Fig. 2B, lanes b and f). The African green monkey total RNA samples exhibited a slightly lower resolution for two bands (Fig. 2B, lane c). These two bands remained when the poly(A^+) RNA fraction was probed, suggesting that there are two distinct mrrRNAs in the African green monkey cells (Fig. 2B, lane d). These results demonstrate that polyadenylated mrrRNAs of roughly 0.2 kb are produced in all the species examined. They confirm the previous results for the presence of polyadenylated mrrRNA in human, and extend the occurrence to rat, mouse, Chinese hamster, and African green monkey. The polyadenylation status of the mrrRNAs obtained previously from rat and mouse (*14*) is not known, but they may contain polyadenylated mrrRNAs. The 0.3-1.3 kb RNA bands in HeLa cell total RNAs detected by the human specific oligonucleotide probe are apparently not polyadenylated and may be equivalent to the D-loop region RNAs reported previously for the rat (*15*).

Cloning and Sequencing of Rat and Human cDNAs—To isolate cDNAs specific for polyadenylated mrrRNAs from rat and human, we synthesized PCR primers as RP2 for rat and P2 for human (see "MATERIALS AND METHODS," Fig. 3, A and B). At the same time, RP1 primer for rat and P1 primer for human with their sequences for an upstream region adjacent to respective LSPs were synthesized. To examine whether mrrRNAs are transcribed from the LSP or the upstream region, we used these four primers together with a 3′-end oligo-dT primer for RT-PCR amplification of mrr cDNAs from JTC-19 and HeLa. The rat RP2 primer produced an amplification product of *ca.* 0.2 kb from polyadenylated RNA of JTC-19 cells. The amplified cDNA product was cloned, designated as mrr/JTC-19-1, and sequenced as shown in Fig. 3A. It contained 158 bases identical to the published mitochondrial sequence (nucleotide numbers 16013 to 16170 in Fig. 3A), including most of the region between the LSP (nucleotide number 16173) and CSB1 (*2*), and 20 adenyl residues at its 3′ end. On the other hand, no amplified products were obtained when RP1 primer was used instead of RP2 (data not shown). These results show that the 5′ end of the original template mrrRNA for this clone is located within a few bases of LSP and most probably at LSP, since RP2 begins three bases downstream from the LSP (indicated by "???" in Fig. 3A). The largest recognizable ORF in the sequence of mrr/JTC-19-1 would encode a peptide of only 26 amino acids with no significant homology to any known protein sequences (Fig. 3A). Since this ORF region is transcribed from L-strand sequences between LSP and OriH, it is different from the reported ORF region present in the D-loop region between OriH and $tRNA^{Phe}$ (*8*). A 12-nucleotide stretch in mrr/JTC-19-1, nucleotide numbers 16084 to 16095 shown in italics in Fig. 3A, is complementary to the 3′-terminal region of rat mitochondrial 12S rRNA (*2, 31*, EMBL X14848 nucleotide numbers 1022-1011, Fig. 4). Our results confirmed the presence of mrrRNA in rat first reported by Sbisa *et al.* (*15*), and revealed its molecular characteristics in further detail.

With the human P2 primer, we isolated a cDNA clone designated as mrr/HeLa-1 from HeLa cell polyadenylated RNA. The cDNA contained 182 bases of template sequence from nucleotide numbers 229 to 406 (Fig. 3B), which

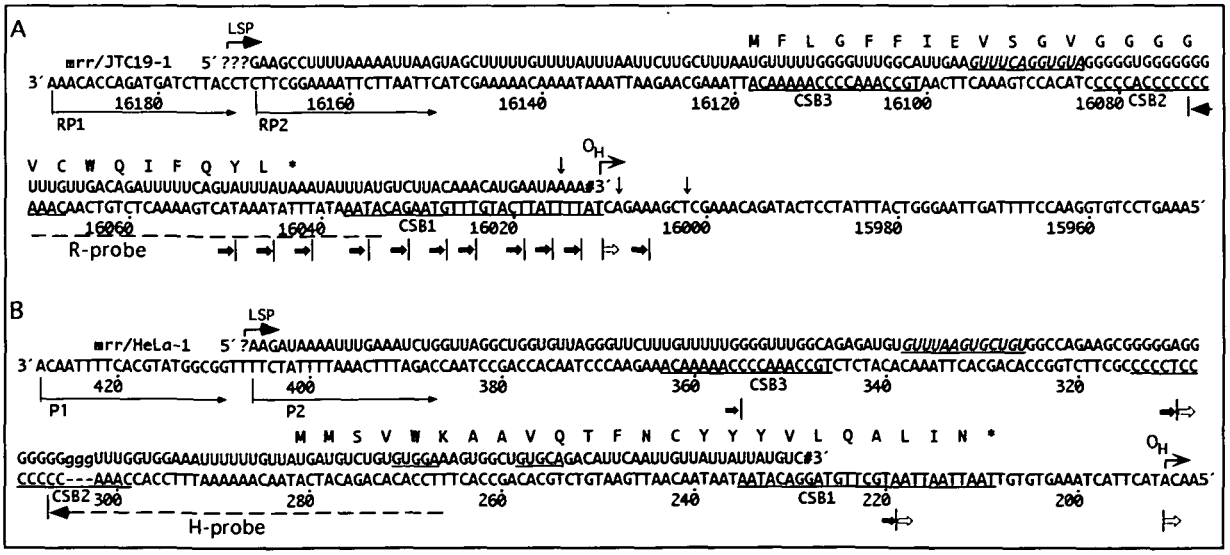

Fig. 3. **Alignment of rat (top) and human (bottom) mrrRNA sequences with their respective mrrDNA sequences.** The rat and human sequences were determined from cDNA clones, mrr/JTC-19-1 (A) and mrr/HeLa-1 (B), respectively. The L-strand reference mrrDNA sequences are also presented for rat (*2*, numbering according to Genbank X14148), and for human (*1*). Also indicated are RP1, RP2, P1, and P2 primers used for RT-PCR (see "MATERIALS AND METHODS"); O_H: origin of H strand replication; →|: 3′-end of mrrRNA; |⇨ : 5′-end of HD-DNA; →|⇨: mrrRNA-DH-DNA linker molecule (*7, 11, 15, 24*); GNGNA: proposed RNase MRP site (*38*); ↓ : *in vitro* RNase MRP cleavage site (*39*); underlined italic letters: proposed 12S rRNA recognition site (*13*); CSB1, 2, 3: conserved sequence blocks (*4*); #: 3′ polyadenylation site for mrrRNA.

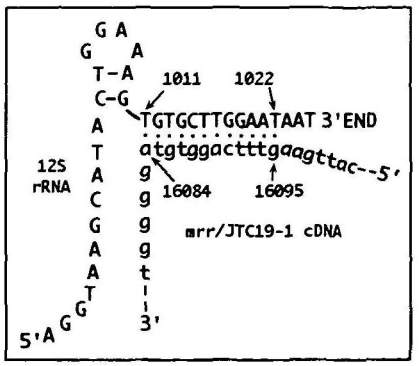

Fig. 4. **Postulated 12-nucleotide complementarity between the 3′-end region of rat 12S rRNA and mrr/JTC-19-1 sequence.** A computer-assisted secondary structure of rat 12S rRNA (upper case letters, EMBL X14848, 2) allowing maximum base-pairing to a 12-nucleotide sequence within the mrr/JTC-19-1 cDNA sequence (lower case letters) is shown. The position of the 12-nucleotide sequence spans from mitochondrial DNA nucleotide numbers 16095 to 16084.

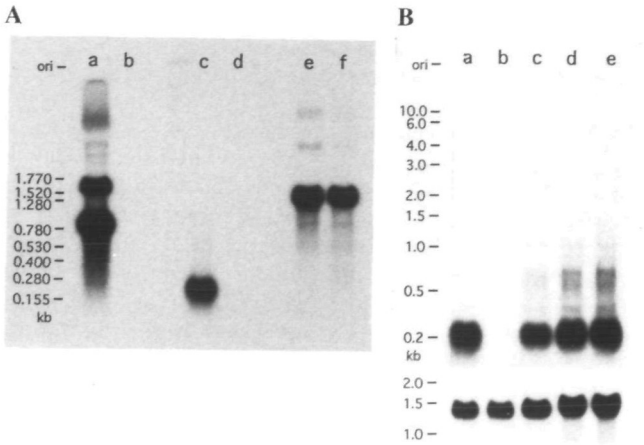

Fig. 5. **Northern blot analyses of the ρ^0 HeLa and the HeEB cybrid cells.** (A) Each lane was loaded with 10 μg of total RNA from HeLa (lanes a, c, and e) or from ρ^0 HeLa cells (lanes b, d, and f), electrophoresed as described, and probed with ^{32}P-labeled DNA probes for mitochondrion-specific CoxIII (lanes a and b), IB43 (lanes c and d), or nuclear-encoded GAPDH (lanes e and f). Size markers indicated on the left are from a 0.16–1.77 kb RNA ladder (Gibco BRL, Maryland, USA). (B) Ten micrograms each of total RNAs from parental HeLa and ρ^0 HeLa cells was loaded in each lane (lanes a and b). Lanes c, d, and e were loaded with 10, 20, and 30 μg of total RNA from HeEB cells, respectively. The blot was hybridized with ^{32}P-labeled IB43 cDNA probe, washed and exposed to X-ray film for 1 h at −80°C (upper panel). The blot was then re-hybridized with ^{32}P-labeled nuclear-encoded GAPDH probe and processed as above (lower panel). Size markers indicated on the left are from a 0.2-10 kb RNA ladder (Novagen, Madison, WI, USA).

included the most of the region between the LSP and CSB1, and 41 adenyl residues at its 3′ end. This size is consistent with the estimated size from Northern analyses (Fig. 1, lane a, Fig. 2, lanes a and b). Again, no amplification products were obtained with P1 primer (data not shown). These results show that the 5′ end of the original template mrrRNA for mrr/HeLa-1 clone is located within a few bases of LSP, and most probably at LSP, since P2 begins one base downstream from the LSP (indicated by "?" in Fig. 3B). They are essentially the same as those described above for mrr/JTC-19-1. The polyadenylation site for the mrr/HeLa-1 RNA was mapped to nucleotide number 229 while the polyadenylation site in the IB43 cDNA maps to the upstream position 237 (*13*, Fig. 3B).

Origin and Intracellular Localization of mrrRNAs—In the first study, we biochemically fractionated HeLa cell homogenates and obtained a mtRNA fraction and a cytoplasmic RNA (ctRNA) fraction for Northern analyses (*32*). Northern analysis using the CoxIII and GAPDH cDNAs (*30*) as probes confirmed that these fractionated RNAs contained minimal cross contaminations. When these RNAs were probed with the IB43 cDNA probe, the mtRNA yielded an intense band of mrrRNA at *ca.* 0.2 kb, while ctRNA gave only a faint band (data not shown). These results suggest that the mrrRNA is primarily localized to the mitochondria. We then isolated total RNAs from the mitochondrial genome-deficient mutant HeLa cell line, ρ^0 HeLa, and its parental HeLa cell line. Northern blot analyses demonstrated that the IB43 cDNA probe did not detect any *ca.* 0.2 kb band in the ρ^0 HeLa total RNA (Fig. 5A, lane d), in contrast to the parental HeLa total RNA (Fig. 5A, lane c). The control CoxIII cDNA probe yielded two intense bands in the parental HeLa RNA sample (Fig. 5A, lane a), but no bands in the ρ^0 HeLa RNA sample (Fig. 5A, lane b), while the nuclear GAPDH probe detected a band in both RNA samples (Fig. 5A, lanes e and f). Finally, to investigate if mrrRNA is recovered in HeEB (a reconstituted cell line obtained by fusing ρ^0 HeLa with enucleated parental HeLa cells), we obtained total RNA of HeEB and examined it for the presence of mrrRNA along with the total RNA samples used in the Fig. 5A as controls (Fig. 5B, upper panel). Here, the mrrRNA band at *ca.* 0.2 kb was detected in the HeEB total RNA by the IB43 cDNA probe (Fig. 5B, upper panel, lanes c-e). Both parental HeLa cell and ρ^0 HeLa cell total RNAs exhibited the same results as seen in the Fig. 5A (Fig. 5A, lane c, and Fig. 5B, upper panel, lane a; Fig. 5A, lane d, and Fig. 5B, upper panel, lane b). The GAPDH probe detected a band at *ca.* 1.5 kb in all lanes, confirming that all lanes contain comparable levels of total RNAs (Fig. 5B, lower panel, lanes a-e). These results clearly show that the occurrence of the detected mrrRNAs is dependent on the presence of a mitochondrial genome, and thus these mrrRNAs are most likely transcribed from the mitochondrial genome.

DISCUSSION

We have confirmed the previous observation that *ca.* 0.2 kb polyadenylated mrrRNAs are present in human cells (*12, 13*) and shown that similar polyadenylated mrrRNAs are present in African green monkey, Chinese hamster, rat, and mouse. These results extend the concept that polyadenylated mrrRNAs are commonly distributed among mammalian species.

We demonstrated, using ρ^0 HeLa cell and HeEB cell total RNAs, that mrrRNAs are produced within the mitochondrial compartment (Fig. 5). Also, in a separate study, we obtained 20 putative mouse mrrDNA-containing genomic clones and one clone from human cells, all of which were found to be fusion products of nuclear and mtDNAs artificially formed during library construction (data not shown). The 7S RNA was purified from a mitochondrial fraction of HeLa cells (*12*). Together, these studies indicate

that mrrRNA is not transcribed from the chromosomally integrated mrrDNA-like sequences, as suggested by others (*13*). Based on our observations, as well as those of others (*13, 19*), we suggest that mrrRNA production in the mitochondria is regulated by inducible nuclear gene(s). Among mitochondrial gene expression factors, mtTF-1 (mtTF-A) is of particular interest here, since the majority of mrrRNAs are transcribed from the LSP (*12, 14, 15*), and since the nuclear coded mtTF-1 activates the LSP (*33*) and is involved in mtDNA replication (*34, 35*).

Analyses of the cDNA sequences for mrrRNAs revealed that CSB sequences are commonly present in human and rat polyadenylated mrrRNAs, and in the previously isolated cDNAs, IB43 and IB65 (*13*, Fig. 3). This is noteworthy because the CSBs are reported to be the recognition sequences for endoribonucleases such as RNase MRP and/or endonuclease G (*36-40*), which are suggested to produce primers for H-strand DNA replication through the cleavage of mrrRNAs (*36*). The polyadenylation site of the mrr/JTC-19-1 cDNA was mapped to two nucleotides 5′ to the OriH, which includes the most of the CSB 1 sequence and one of the three *in vitro* endonuclease cleavage sites indicated by downward arrows in Fig. 3A (*39*). This cDNA also includes 7 of the 11 putative processing sites (*15*) located within CSB1 (Fig. 3A). However, the human mrr/HeLa-1 cDNA polyadenylation site maps to nucleotide number 229. Therefore, this cDNA overlaps with CSB1, while cDNAs IB43 and IB65 do not (*13*). This suggests the involvement of sites other than CSB1 in 3′ end formation. Sequence analyses revealed that all three human cDNAs include CSB II and CSB III, which are shown to be essential for mitochondrial endoribonuclease cleavage in mouse and human (*37, 40*), and at least one of two 5′-GNGNA-3′ sequences that have been proposed to be the target sites for the RNase MRP (*38*). Although polyadenylated RNAs do not serve directly as primers for DNA replication (reviewed in Ref. *41*), the above structural characteristics of polyadenylated mrrRNAs suggest they are candidate substrates for endoribonucleases. A recent review states that an extremely stable R-loop (a structure consisting of two DNA strands and one RNA strand) can be formed using a CSB II-containing RNA fragment, and that the substrate for RNA processing is probably the triple-stranded RNA-DNA hybrid (*42*). Further study should incorporate such structural aspects.

We also found that the mrr/JTC-19-1 cDNA clone contains an apparent 12S rRNA binding region and an ORF for a short peptide. These structural features support the previously proposed hypotheses that polyadenylated mrrRNAs are multi-functional molecules, which serve as primers for H-strand DNA synthesis, code for a peptide, and regulate translational processes through rRNA binding site (reviewed in Ref. *41*). On the other hand, since the human mrr/HeLa-1, IB43, and IB65 cDNAs do not include any recognizable ORFs or extend to OriH, the possibility remains that these small polyadenylated mrrRNAs function in different ways, and that longer molecules analogous to mrr/JTC-19-1 are yet to be isolated from human cells.

Two distinct types of polyadenylated mrrRNAs were demonstrated in African green monkey. The monkey mrrRNAs of different sizes may be produced from either of two presumptive LSPs found within the unique triplication of an 108 bp sequence in the mrrDNA in this species (*17*). These RNAs could be suitable subjects for studying differential gene expression as well as the functional and the structural heterogeneity of mrrRNAs.

The nuclear control of mitochondrial function has been related to development and aging (*27, 43*), yet the mechanisms are essentially unknown. The cDNA clones obtained in the present study will serve as useful tools in furthering this attractive field of science.

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