The Transcription of DNA in Chicken Mitochondria Initiates from One Major Bidirectional Promoter*

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The mitochondrial genome (mtDNA) of vertebrates is a small, compact, closed circular molecule of about 16 kilobases containing an identical set of 37 genes specifying 13 proteins, two rRNAs and 22 tRNAs encoded in both the heavy (H) and light (L) DNA strands (see Ref. 1 for review). The gene organization is the same in all eukaryotic mtDNAs sequenced thus far, except for birds (2). Size variation among vertebrate mtDNAs is mainly due to the number of nucleotides in the control region of each species. This region, the major noncoding segment of vertebrate mtDNAs, spans the area between the genes for tRNA^Glu in birds and tRNA^Arg in mammals, and contains the H-strand replication origin and the promoters for both the H- and L-strands (see Ref. 3 for a review). In mammalian and amphibian mtDNAs (4–9), the H-strand transcriptional initiation site is located within 35 nucleotides upstream of the gene for tRNA^Met, and ~70–120 base pairs downstream of the L-strand start site. Both human and mouse mtDNAs have independent, predominantly unidirectional promoters for transcription of the individual strands (10–12). Important bidirectional transcription in these species is observed only in vitro, whereas in vivo the opposite strand is transcribed at a much lower rate (13). In contrast, the major promoter identified in Xenopus laevis has been shown to be fully bidirectional in vitro, while a minor promoter predominately rises to L-strand transcripts and only exerts weak bidirectionality in vivo (9, 14, 15). Bidirectional transcription in X. laevis mtDNA appears to result from the symmetrical arrangement of a consensus sequence within the promoter region (9).

As mitochondrial transcription initiation revealed important differences between amphibia and mammals, we investigated which type of mitochondrial promoters prevails in chicken. The localization of H- and L-strand initiation sites was assessed by S1 nuclease protection and primer extension analysis and by the sequencing of mtRNA transcripts capped in vitro by the vaccinia virus guanylyl transferase. The results obtained indicate that transcription of both mtDNA strands initiates from one major bidirectional promoter, reminiscent of the situation in amphibia.

MATERIALS AND METHODS

Isolation of Liver Mitochondria—Liver mitochondria from white Leghorn chickens were obtained by differential centrifugation as described previously (16), and further purified on a discontinuous sucrose gradient according to Bogenhagen and Clayton (17). The mitochondrial fraction collected from the gradient was diluted with two volumes of water and centrifuged. The pellet was resuspended in homogenizing buffer, quick-frozen on dry ice and kept at ~70 °C until used.

Preparation and Capping of mtRNA—Mitochondria were lysed by the addition of 2% Sarkosyl and nucleic acids were isolated by repeated phenol-chloroform extractions. Following ethanol precipitation, the preparations were enriched in high molecular weight mtRNAs by LiCl treatment (18). RNA was then ethanol-precipitated repeatedly, dissolved in water and stored at ~70 °C.

MtRNA (300 µg) was lyophilized and resuspended in 11 µl of 5 mM methylmercuric hydroxide (19). It was capped in vitro using 230 µCi of [α-32P]GTP (Amersham Corp., 3000 Ci/mmol) and 20 units of vaccinia virus guanylyl transferase. The capped RNA was then ethanol-precipitated repeatedly, dissolved in water and stored at ~70 °C.

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Transcription in the Chicken Mitochondria

RNA Sequencing—Capped mtRNA, partially digested by RNase T1, was loaded on a 15% polyacrylamide 7 M urea gel. After electrophoresis, bands detected by autoradiography were excised from the gel and extracted in water for 40 h at room temperature. The eluted material was desalted on Sephadex G-50 and lyophilized. Enzymatic RNA sequencing was performed according essentially to the method of Donis-Keller et al. (20), with some minor modifications as published previously (16).

Filter Hybridization—Plasmid DNA inserts (100–300 ng) covering the entire chicken mitochondrial genome, or single-stranded M13 phage DNA (1 µg) containing mtDNA segments encompassing the D-loop region or part of it, were run in 0.7% agarose gels and alkaliblotted to Hybond-N membranes (Amersham Corp.). Filters were hybridized in formamide solutions at 42 °C for 2 days with capped mtRNA as a probe. RNA was denatured for 5 min at 50 °C prior to hybridization. Prehybridization, hybridization and washing conditions were as specified by the membrane supplier. Autoradiography was performed at ~70 °C for 2 weeks with intensifying screens.

Primer Extension Analysis—mtRNA (100 µg) was treated with 100 units of DNase I (RNase-free; Pharmacia LKB Biotechnology Inc.) at 20 °C for 10 min. The products of RNAguard (Pharmacia LKB Biotechnology Inc.). After a phenol-chloroform extraction, the RNA was ethanol-precipitated and dissolved in water.

Synthetic probes used for primer extension analysis were synthesized with an automated system (GeneAssembler; Pharmacia LKB Biotechnology Inc.). Oligonucleotides were 5' end-labeled using T4 polynucleotide kinase (Pharmacia LKB Biotechnology Inc.) and [-32P]ATP (Amersham Corp., 5000 Ci/mmol), and purified on 20% polyacrylamide, 7 M urea gels. The excised bands were extracted overnight at room temperature in 1 mM EDTA, pH 8.0, and the eluted material was desalted on Sephadex G-50. Labeled DNA probes (0.02 pmol) were annealed to 10 µg of DNase-treated mtRNA in 10 µl of a heating buffer containing 120 mM KCl and 10 mM Tris-HCl, pH 8.5 (15). After hybridization, samples were chilled on ice, and 10 µl of cold reverse transcriptase buffer (120 mM KCl, 100 mM Tris-HCl, pH 8.5, 20 mM MgCl2, 10 mM dithiothreitol, 1.6 mM of each of four dNTPs, 25 µg/ml actinomycin D) was added, plus 10 units of avian myeloblastosis virus reverse transcriptase (Pharmacia LKB Biotechnology Inc.) and 25 units of RNAguard. After a 2-h incubation at 42 °C, the extension products were ethanol-precipitated and resuspended in 80% formamide containing 20 mM EDTA and dyes. The mixture was then boiled for 2 min and electrophoresed on a 6% polyacrylamide, 8 M urea sequencing gel in 90 mM Tris-borate, pH 8.3, 2 mM EDTA buffer. Gels were fixed and dried prior to autoradiography. The samples were run alongside sequencing ladders of M13 recombinant templates containing L- or H-strand mtDNA fragments of the D-loop region primed with the above oligonucleotides. Sequencing reactions were performed with modified T7 DNA polymerase (Sequenase, United States Biochemicals Corp.) and [-32P]dATP (Amersham Corp., 1000 Ci/mmol).

S1 Nuclease Protection Assays—Plasmid pMtc5, which contains nucleotides 16477–1737 of the chicken mitochondrial genome (2), was used to prepare the S1 probes. The sequence contains all or part of the following genes: 12 S rRNA, tRNA^{Ψ}, the control region, tRNA^{Ψ}, and ND6. Long restriction fragments were dephosphorylated and 32P-labeled at their 5' ends with T4 polynucleotide kinase (Pharmacia LKB Biotechnology Inc.). After further restriction, the smaller fragments uniquely labeled on only one strand were fractionated on polyacrylamide/urea gels. The 5' end-labeled single strands were cut out of the gel, eluted in 1 mM EDTA, and desalted on Sephadex G-50. The identity of the single strands used as probes was confirmed by DNA sequencing. Hybridization of S1 probes to chicken mtDNA (5–10 µg) was performed in 10 µl of 80% formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4, and 1 mM EDTA. After 15 min at 72 °C, the mixtures were incubated for 3 h at the appropriate Tm. After hybridization, the reaction mixtures were diluted with 200 µl of ice-cold S1 buffer containing 0.25 M NaCl, 0.05 M sodium acetate, pH 4.6, 4.5 mM ZnCl2, 100 units of S1 nuclease (Boehringer Mannheim). Reactions were incubated at 37 °C for 30 min and stopped by the addition of two volumes of chilled ethanol and 12.5 µg of yeast tRNA carrier. Samples were boiled and electrophoresed on polyacrylamide/urea gels. The protected fragments were electrophoresed with sequencing ladders of M13 recombinant templates containing L- or H-strand mtDNA fragment of the control region, primed with the oligonucleotides used in the primer extension reaction.

RESULTS

Capped Transcripts of Chicken mtDNA Map Primarily to the Control Region—Transcription initiation sites of chicken mtDNA were mapped by methods successfully used in yeast and a number of vertebrate species (4, 5, 9, 19, 22). MtDNA primary transcripts which are not capped in vitro (21) can be capped in vitro by the vaccinia guanylyl transferase and [γ-32P]GTP. Labeled transcripts were used as a probe in hybridizations with filter-bound restriction fragments of mtDNA. Fig. 1 shows that in chicken-capped mtRNA transcripts hybridize primarily to the control region. Faint hybridization to fragments not containing the control region was also detected indicating the presence of some other capppable RNA species in the mtRNA preparations. As already suggested for X. laevis (9), these faint bands may reflect either hybridization of long stable precursor transcripts initiated within the control region or transcripts initiated in other regions of the chicken mitochondrial genome. Alternatively, mtRNA species with capable 5' ends could be generated through the maturation process of H- or L-strand transcripts, as documented for chicken mitochondrial tRNA^{Ψ} (16).

To determine more precisely from which part of the control region the capped RNA species are derived, we hybridized

![Fig. 1](image-url)
capped RNA to single-stranded DNA prepared from M13 subclones covering that region (Fig. 2). Strong signals appeared only with the H-strand mtDNA (Fig. 2A), suggesting a significantly higher steady-state level of primary H-strand transcripts than that of the L-strand. No hybridization was found with H-strand subclone 10, which contains a part of the 12S rRNA gene exclusively. The signal intensity obtained with subclone 9, which does not contain any control region sequences, was substantially weaker than that for subclone 8, suggesting that transcription starts on the H-strand.

Strong signals appeared only with the H-strand mtDNA (Fig. 2). A weak hybridization was detected in lanes 11 to 14 suggesting that transcription starts on the L-strand between the H-strand and the 12S rRNA gene exclusively. The signal intensity obtained with H-strand subclone 10, which contains a part of the 12S rRNA gene, was substantially weaker than that for subclone 8, suggesting that transcription starts on the H-strand.


Fig. 2. Hybridization of capped mRNA to DNA fragments in the control region. A and B, single-stranded phage DNAs (M13mp8 or mp19) containing the entire or part of the control region, were hybridized to in vitro capped mRNA. In A, lanes 3–10 correspond to phage DNAs containing mtDNA H-strand sequences. Lanes 1 and 2 correspond to the M13mp19 and M13mp8 vector DNA respectively serving as controls. In B, lanes 11–18 correspond to phage DNAs containing mtDNA L-strand sequences. The length, position, and orientation of the mtDNA inserts of the phages are outlined in C. The bold middle line represents the control region contained within mtDNA fragment 8 (Fig. 1B). tRNA^"m^" (F) and tRNA^"g^" (E) genes, and part of the 12S rRNA and ND6 genes are included. Nucleotide 1 marks the 5′ end of the control region, immediately adjacent to the 3′ end of the tRNA^"m^" gene at nucleotide 16,775 (2). 12S rRNA and tRNA^"m^" genes are H-strand-encoded, whereas tRNA^"g^" and ND6 genes are L-strand-encoded. The arrows indicate the direction of transcription of the H- and L-strands, respectively. Numbers on each side of panel C refer to the phage DNAs in A and B. Each horizontal line represents the length (to scale) of the H- or L-strand mtDNA sequence contained within the respective phage DNA.

Fig. 3. RNA sequencing of a H-strand transcript digested by different enzymes. MtRNA was capped in vitro and partly digested with RNase T1. A 32-nucleotide product was isolated and sequenced. The following base-specific ribonucleases were used: T1 (G-specific), U2 (A-specific), Phy M (A+U-specific) and B. cereus (C+U-specific). L, RNA ladder; BPB, bromphenol blue.
Table I

<table>
<thead>
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<th>Description of the primers and probes</th>
<th>Length (nucleotides)</th>
<th>Nucleotide position&lt;sup&gt;a&lt;/sup&gt;</th>
<th>5' end&lt;sup&gt;b&lt;/sup&gt;</th>
<th>3' end&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>Extension primers</td>
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<td>H1</td>
<td>25</td>
<td>1229</td>
<td>1205</td>
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<tr>
<td>B</td>
<td>305</td>
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<sup>a</sup> Nucleotide numbers refer to Fig. 7.

<sup>b</sup> All probes and primers are 32P labeled at their 5' end.

Fig. 4. Primer extension mapping of 5' ends of H-strand transcripts using probe H1. A, the 5' end-labeled single-stranded primer H1 (see Table I) was annealed to DNase-treated mtRNA and extended using avian myeloblastosis virus reverse transcriptase. Lanes: 1, no mtRNA; 2, 10 μg of mtRNA; G, A, T, and C, a dideoxynucleotide sequence obtained by extending probe H1 on M13 phage DNA subclone 11 (Fig. 2C). Positions of the unextended primer and that of the major extension product a are shown by arrowheads. B, longer migration of the samples in A, for a more precise mapping of the major extension product a. Lane description is as in A.

identified by the use of a sequence ladder run in parallel and obtained by extension of primer H1 on M13 subclone 11 (see Fig. 2C). Multiple origins at H-strand transcription sites have also been reported for human (5) and bovine (8) mtDNAs, although, in all cases, the heterogeneity observed could be due to incomplete primer extension near the 5' end of the RNA template.

Besides the major extension products, multiple weakly labeled products are seen which run faster than band a (Fig. 4A, lane 2). The large number of these products suggests that they arose from site-specific pausing of the reverse transcriptase or from extension of RNA templates from degraded primary transcripts. A further extension experiment was performed using primer H2 whose sequence spans the segment between the 5' end region of the 12 S rRNA gene and the adjacent 3' end region of the tRNA<sup>Phe</sup> gene (see Table I and Fig. 7). These results confirm those obtained with primer H1. Upstream of the 5' end of the tRNA<sup>Phe</sup> gene (band b in Fig. 5A), a series of extended products are detected at positions which correspond to those seen in Fig. 4A. The most intense one has a 5' terminus at nucleotide 1272 ± 3 (band c). S1 nuclease assays using 5' end-labeled probe A (see Table I) confirmed these results. Two protected fragments are seen, one ending at the 5' terminus of tRNA<sup>Phe</sup>, at nucleotide 1229 ± 3 (Fig. 5B, band b), the other at nucleotide 1027 ± 3 (band c). In addition to the latter band, other closely spaced bands are detected in this region of the gel and may represent S1 nuclease nibbling at the A+T-rich end of the RNA-DNA hybrids (23). After prolonged exposure of the filters, very faint bands are detected upstream of the gene for tRNA<sup>Phe</sup>, at positions that coincide with those seen with primers H1 and H2. These observations suggest that a H-strand transcript is initiated at nucleotide 1072 on chicken mtDNA and extends into tRNA<sup>Phe</sup> and likely into 12 S rRNA as well. This primary transcript is processed at the 5' terminus of tRNA<sup>Phe</sup> and possibly at other positions between tRNA<sup>Phe</sup> and the initiation site of the transcript.

A few extended products migrate between primer H2 and band b (Fig. 5A). The most intense one, designated a, maps at position 1238, which correspond to the first nucleotide of the tRNA<sup>Phe</sup> D-stem. Protected fragments are seen at those positions in S1 nuclease assays, the most intense of which maps at nucleotide 1238 (Fig. 5B, band a). These observations suggest the possibility that an additional transcription initiation sites resides in the H-strand. Alternatively, small, proc-
essed tRNA\textsuperscript{Phe} transcripts could account for the results obtained.

5' End Mapping of L-strand Transcripts—Results from the hybridization of capped RNAs to single-stranded mtDNA fragments (Fig. 2B) showed that the L-strand transcriptional origin is located in the same region as that of the H-strand. Primer extension analyses further delimited this region to a few nucleotides on the L-strand DNA segment facing the H-strand transcriptional origin. Using primer L1 (see Table I and Fig. 7) to prime the extension reaction, a major product is detected, the 5' end of which maps at nucleotide 1071 ± 1 (Fig. 6A, band a). A few minor products appeared as well which are a few bases longer than the major one. Similar results were obtained using primer L2 (data not shown) whose sequence is complementary to part of the putative CSB1 (see Table I and Fig. 7). Other faint bands are seen above those at nucleotide 1071 ± 2, at the same position as that of the major product extended from primers L1 and L2.

DISCUSSION

Our data show that in chicken the major sites for transcription initiation of both the H- and L-strands reside in the control region of the molecule. The start sites are located upstream of the gene for tRNA\textsuperscript{Phe}, similar to the situation in amphibia and mammals. Hybridization of capped mtRNA to filter-bound H- and L-strand DNA suggests that the steady-state level of the H-strand transcripts in chicken mitochondria is higher than that of the L-strand. This view is also supported by primer extension experiments using H- and L-strand-specific oligonucleotides as primer. These results are in contrast to those reported for amphibia and mammals where capable L-strand primary transcripts are more abundant than those of the H-strand (7, 9). The different levels of primary H- and L-strand transcripts in chicken can be due to differences in the promoter strength or in the processing rate of the nascent transcripts.

In amphibia and mammals, the major H-strand initiation site resides within a 35-nucleotide stretch immediately preceding the tRNA\textsuperscript{Phe} gene. This location contrasts to that in chicken where this site maps 156 bases upstream of the tRNA\textsuperscript{Phe} gene. Sequence analysis of this interval reveals the presence of a potential open reading frame coding for a polypeptide 26 amino acids long using ATG as the initiation codon. Furthermore, there are two stretches of 11 nucleotides each upstream of the open reading frame with a 10 out of 11 match to two complementary sequences in the 3' end region of chicken 12 S rRNA (Fig. 8A). These sequences are not found elsewhere in the chicken mitochondrial genome. The corresponding RNA is 155 nucleotides long, contains a universal stop codon encoded in mtDNA, and is relatively abundant as judged by primer extension analysis. A similar RNA is found in human HeLa cell mitochondria and has been termed "7 S RNA" on the basis of its sedimentation coefficient (24). The 7 S RNA, which also contains a universal stop codon encoded in mtDNA, is polyadenylated and is the most abundant poly(A)-containing mtRNA in HeLa cells. It also contains an open reading frame for a potential protein of 23 or 24 amino acids and an 11-nucleotide sequence is present near its 5' end that is complementary to the 3' end of human 12 S rRNA. A portion of the 7 S RNA is found associated with the mitoribosomes, but it is not known whether the 7 S RNA is translated. On the other hand, the non-polyadenylated 7 S RNA in human as well as related sequences in other vertebrates (25, 26) are believed to prime both the replication
of the H-strand and transcription of the L-strand. This would likely not be the case for the putative chicken H-strand 7S RNA-like sequence.

In human mitochondria, a capped H-strand transcript species has been mapped about 30 nucleotides upstream from the 3' end of the structural tRNA\(^\text{Ph}^\text{m}\) gene (4). In vitro studies of transcription kinetics in human HeLa cells have added to this finding suggesting the presence of an additional H-strand promoter in the tRNA\(^\text{Ph}^\text{m}\) gene (27). This promoter is believed to initiate the transcription of almost the entire H-strand. The other, more active promoter, located just upstream of the tRNA\(^\text{Ph}^\text{m}\) gene would initiate the transcription of a polycistronic molecule that terminates at or near the 3' end of the 16S RNA. In bovine and X. laevis mitochondria, primer extension experiments have indicated the presence of an additional transcription initiation site in the structural gene for tRNA\(^\text{Ph}^\text{m}\) (8, 9). However, S1 nuclease assays failed to confirm these results and suggested that the extended products arose through site-specific pausing of the reverse transcriptase in the RNA templates. In chicken, both primer extension and S1 nuclease protection give bands which map within the structural gene for tRNA\(^\text{Ph}^\text{m}\) at the same positions as in bovine and X. laevis. As no chicken-capped RNA species mapping in this region has been sequenced, it is difficult to conclude that an additional H-strand transcription start site is present in the gene for tRNA\(^\text{Ph}^\text{m}\).

The major L-strand transcriptional initiation site maps 1 base pair from that of the H-strand. As in the case of the H-strand, initiation occurs within the space of a few nucleotides, but there is no evidence that L-strand transcription initiates at an alternate position in the control region. It has been shown in other vertebrates that the L-strand start site serves both for the transcription of the L-strand, and for the replication of the H-strand. The origin of the H-strand in chicken is located in the immediate vicinity of CSB1 at nucleotides 870–892 (2), a distance from the L-strand start site about the same as that seen in other vertebrates (~170 nucleotides). Contrary to the situation in human mitochondria, no open reading frame is found within this sequence.

The sequence encompassing the bidirectional promoter in chicken mitochondria contains an almost perfect inverted repeat with a 16 out of 18 match (Fig. 8B). We suggest that the bidirectional character of the chicken mitochondrial promoter results from the symmetrical arrangement of this sequence which can be folded into a cruciform structure (Fig. 8C), with the major H- and L-strand initiation sites positioned into the loops. In X. laevis, bidirectional transcription appears also to result from the symmetrical arrangement of a consensus sequence within the promoter region (9). The sequence is eight nucleotides long (5'-ACGATAAA-3'), surrounds the start site, and is an essential promoter element (15). This octanucleotide presents a six or seven out of eight match with two sequences comprised within the chicken inverted repeat. One of the octanucleotide (5'-ACATTATT-3') flanks the 3' end of the H-strand initiation site and the other (5'-ACGATAAA-3') that of the L-strand. A similar motif is found in the H-strand promoter region in mouse. Computer searches for matches to the octanucleotide at other sites within the chicken mtDNA sequence revealed three locations. However, none of these sites are flanked by an A + T-rich sequence similar to that found in the control region inverted repeat.

Chicken mtDNA is thus far unique in having one major bidirectional promoter. Other vertebrates have either several bidirectional (X. laevis) or two, mainly unidirectional (mouse, cow, human), promoters. Mitochondrial transcription studies of further bird species as well as reptilia will be needed to determine whether this arrangement has occurred in the vertebrate lineage giving rise to birds or whether it is more ancient.

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