Mapping of Control Elements in the Displacement Loop Region of Bovine Mitochondrial DNA*

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Thomas C. King‡ and Robert L. Low

From the Departments of Pathology and Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110

The genomes of mammalian mitochondria are duplex DNA circles. The two major transcriptional promoters and the origin of DNA replication for one DNA strand are located in a single region which contains no structural genes and occupies about 6% of the genome. This region is called the displacement loop (D-loop) region since it is often found as a novel triplex structure in which the heavy strand of the genome has been partially replicated. This nascent single-stranded DNA segment remains hybridized to the light strand, displacing the heavy strand of the genome over much of the D-loop region. The promoters and the sites of initiation of D-loop DNA synthesis have been mapped in the human and mouse genomes and may show limited sequence conservation. We have mapped these sites in the bovine mitochondrial genome. Some features are conserved between all three species; however, the promoters and the sites of initiation of D-loop DNA synthesis show no primary sequence homology among all species. This lack of sequence homology is in contrast to the greater than 80% sequence conservation which has been reported in portions of the D-loop region which are located distal to the origin of DNA replication and far from the transcriptional promoters. These results imply that closely related species may have developed different means of controlling mitochondrial gene expression.

The mitochondrial genome of mammals is composed of a small (~16 kilobases) duplex circular DNA which encodes select polypeptides of the inner membrane and the RNA components of the mitochondrial translational system (1). The complete nucleotide sequences of the human, bovine, and mouse mitochondrial genomes have been determined (2–4). They are remarkable both for the extreme evolutionary conservation of their structural genes and for the lack of conservation within their displacement loop (D-loop) regions (which do not subserve a protein-coding function; Ref. 5). The D-loop region is located between the genes for tRNAs Phe and Pro, accounting for approximately 6% of the genome. It contains the origin for heavy-strand DNA replication (OriH) as well as the two promoters (P₁ and P₃) from which polycistrionic mRNAs are transcribed from the heavy (H) and light (L) strands of the duplex genome (6, 7).

A substantial proportion of mammalian mitochondrial genomes contain D-loops (>75% in mouse L cells (8)). These D-loops are triplex structures in which a single-stranded DNA segment (complementary to the light strand of the D-loop region with its 5' end located at OriH) is hybridized to the circular mitochondrial genome, displacing the heavy strand from the light strand in the D-loop region (for a review, see Refs. 9 and 10). These triplex D-loops are ubiquitous in vertebrate mitochondria (10), are fairly rapidly recycled (11), and differ in frequency between replicating and nonreplicating mitochondria (12, 13). Their function is unknown, although they are thought to have a role in the control of mtDNA replication and/or transcription.

Discrete, rather tightly conserved segments of sequence homology have been identified in the human, mouse, and bovine D-loop regions (2). These sequences (~20–40 nucleotides) are not located near the control elements (OriH, P₁, and P₃) mapped in the mouse and human D-loop regions, although they are included in the triplex D-loop structures. Three somewhat less well conserved regions of sequence homology found in mouse, rat, and human mtDNAs (conserved sequence blocks, CSB1–3) are more closely associated with P₁ and OriH (14). Sequences homologous to these are not obvious in the bovine D-loop region, however (Table 1). The most extensive homology is found between CSB2 (14) and the tract of 12 cytosine residues immediately preceding tRNA Phe in the bovine genome, at a point far from P₃ or OriH (see “Discussion”).

Until recently, bovine D-loops had been characterized only by electron microscopy (15). The locations of bovine D-loop 3' ends have been reported recently (16), but precise mapping of D-loop 5' ends and of the transcription start sites has not been reported. We have located these control elements in the bovine mitochondrial D-loop region with the expectation that their localization will facilitate understanding of the features which control transcription and DNA replication in mammalian mitochondria. Although similarities of organization and control are evident between bovine and mouse mitochondria or between bovine and human mitochondria, few features are conserved among all three species. These results suggest that closely related species may have developed rather different means of controlling mitochondrial gene expression and DNA replication.

MATERIALS AND METHODS

Cloning of Bovine Mitochondrial DNA—A crude preparation of bovine mitochondria was isolated from the heart of a castrated male Holstein steer. One kg of ventricular tissue was diced, minced, and homogenized in a Waring blender in 2 liters of buffer containing 30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM sucrose, 2 mM dithiothreitol, 0.2 mM Na₂EDTA, and 1 mM phenylmethylsulfonyl fluoride. Mitochondria were isolated by differential centrifugation in the same
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buffer, essentially as described (17). mtDNA was isolated by lysing the mitochondrial fraction with 2% SDS at 37 °C. NaCl was added to a final concentration of 1 M and the lysate was incubated for 1 h at 0 °C. Insoluble material was pelleted by centrifugation for 15 min at 35,000 × g. CaCl₂ was added to the supernatant fraction, and covalently closed superhelical mtDNA was purified on two successive CsCl density gradients (18). The DNA was extracted with isobutyl alcohol, dialyzed, and ethanol-precipitated. The resulting purified DNA was digested with restriction endonucleases EcoRI and Clal (New England Biolabs) and ligated into a pBR322 vector which had been digested with the same restriction enzymes and treated with calf intestine alkaline phosphatase (Boehringer Mannheim) as described (18). Ampicillin-resistant colonies were isolated and minilysates digested with various restriction enzymes to assess the nature of the DNA cloned. A clone was isolated which contained the EcoRI-ClaI restriction fragment (encompassing nucleotides 12412-990; Ref. 2). This plasmid, designated Rov 1, was used for the construction of all S1 nuclease and primer extension probes described, as well as for dot-blot hybridization assays.

Isolation of Bovine Heart Mitochondrial Nucleic Acids—Bovine nuclear acid samples for S1 nuclease and primer extension assays were prepared from the preparations of bovine heart mitochondria described above. Nucleic acids were purified by first extracting the mitochondrial fraction (500 mg of mitochondrial protein) with lysis buffer containing 50 mM Tris-HCl, pH 7.4, 0.5% SDS, and 50 mM NaCl. Protease (Boehringer) was then added to a final salt concentration of 50-100 μg/ml, and the lysate was allowed to digest overnight at 40 °C. Nucleic acids were isolated by phenol/chloroform (1:1) extraction, chloroform extraction, and ethanol precipitation. The resulting nucleic acid preparation was used in subsequent experiments. These preparations still contained contaminating nuclear DNA as judged by a comparison of the total DNA concentration with the mtDNA concentration estimated by dot-blot hybridization (see below; data not shown). Contaminating nuclear DNA did not impede subsequent analysis.

Growth of Bovine Tissue Culture Cell Lines—Two bovine cell lines were obtained from the American Type Culture Collection. The first, CCL22, is an established cell line (passage approximately 120) isolated from the kidney of a normal steer. The cells are thought to be of epithelial origin as judged by morphology (19). These cells were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum plus penicillin and streptomycin. The other bovine cell line used, CCL209, is a primary culture (passage 16-18) derived from the pulmonary artery endothelium of a young female cow. These cells are endothelial as judged by morphology and were grown in Earle's medium supplemented with 20% fetal bovine serum plus penicillin and streptomycin. All cells were grown in Falcon tissue culture flasks (75 cm²) at 37 °C in an atmosphere of 10% CO₂ at 37 °C. All experiments were pulsed with [3H]thymidine (Amersham Corp.), at 5 μCi/ml for 1 h before harvesting. Total nucleic acids were isolated from tissue culture cells by decanting the media and rapidly adding lysis buffer plus proteinase K to the cells (see above). "Growing" cells were harvested when the cells covered less than 50% of the surface area of the culture flask, whereas "confluent" cells were maintained at confluence for at least 3 days prior to harvesting. CCL22 cells showed a 100-200-fold decrease in [3H]thymidine incorporation on becoming confluent, whereas CCL209 cells showed only a 10-fold reduction (data not shown). Nucleic acids were purified as described for bovine heart nuclear acids.

Dot-blot Hybridization—In order to standardize the nucleic acid preparations from bovine heart and tissue culture cells, the mtDNA concentrations of each sample was assessed using dot-blot hybridization (20). Samples were denatured with alcalase, neutralized, and heated to 100 °C. The samples were chilled on ice and spotted either directly onto nitrocellulose filters (pretreated with 20 × SSC) or after serial 2-fold dilution in H₂O containing 2 μg/ml yeast carrier RNA (Sigma). After drying and baking at 80 °C for 2–3 h the filters were prehybridized for 1 h at 43 °C in 50% formamide, 1 × Denhardt's solution (18), 29 mM sodium phosphate, pH 6.5, 0.2% SDS, 4 × SSC, and 0.1% triton X-100. Hybridization was continued overnight at 43 °C. Filters were washed twice in 2 × SSC, 0.1% SDS, 2 mM Na₂EDTA at room temperature and twice in 0.1 × SSC, 0.1% SDS, 2 mM Na₂EDTA at 40 °C. The filters were then dried and autoradiographed using XAR5 x-ray film (Kodak) and Quanta III intensifying screens (Du Pont).

In each experiment the concentration of mtDNA in the samples was visually judged relative to isodense spots from serial dilutions of Bov 1 DNA spotted on the same filter (see Fig. 1). For convenience, the mtDNA concentration in each sample was calculated by comparison to the hybridization signal from Bov 1 DNA using a conversion factor of 6 (approximately one-half of the Bov 1 plasmid is composed of mitochondrial sequences) (see "Materials and Methods"). The mtDNA sequence contained in Rov 1 is significant). In order to determine whether this phenomenon could lead to significant errors, various mitochondrial nuclear acid samples were treated extensively with RNase A (Sigma) (30 min at 60 °C with 500 μg/ml RNase A) prior to dot-blot hybridization (21). As shown in Fig. 1, prior RNase treatment had little effect on the hybridization signal of these samples.

Since the probe DNA used to estimate mtDNA concentrations includes the entire D-loop region, differences in the fractions of mitochondrial genomes containing D-loops (12, 13) could affect the measurement of the relative mtDNA concentrations in different samples. However, since the proportion of cloned sequences corresponding to D-loops is small (only about 20% of the total), this source of error should be insignificant relative to the accuracy of the technique (2-fold dilutions). Another possible source of error could arise if the amount of 12 S RNA varied between samples (as the amount of 12 S rDNA sequence contained in Bov 1 is significant). In order to determine whether this phenomenon could lead to significant errors, various mitochondrial nuclear acid samples were treated extensively with RNase A (Sigma) (30 min at 60 °C with 500 μg/ml RNase A) prior to dot-blot hybridization (21). As shown in Fig. 1, prior RNase treatment had little effect on the hybridization signal of these samples.

Fig. 1. Dot-blot hybridization. A shows the result of dot-blot analysis of serial 2-fold dilutions of Bov 1 plasmid DNA beginning with yeast carrier RNA. At the end of prehybridization, each DNA solution was deposited in each spot. B shows the result of serial dilutions (from the same experiment) for six different CCL22 cell nuclear acid preparations with (right) or without (left) prior RNase treatment (see "Materials and Methods"). Calculated final mtDNA concentrations were: 1 and 2, 0.23 μg/ml; 3, 0.47 μg/ml; and 4 and 6, 1.88 μg/ml; and 5, 2.81 μg/ml.

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S1 Nuclease Analysis—S1 nuclease assays were performed on mitochondrial nucleic acid samples from bovine heart and tissue culture cells as previously described (22) except that all samples were heated to 100 °C for 15 s prior to hybridization at 65 °C (in order to denature the double-stranded mtDNA samples). RNA-DNA and DNA-DNA hybridization was carried out in 25 μl of sequestering buffer containing 25 mM HEPES, pH 7.8, 400 mM NaCl, and 1 mM Na2EDTA. After hybridization, samples were chilled on ice, and 200 μl of cold S1 nuclease buffer (30 mM NaAc, pH 4.7, 250 mM NaCl, 5% glycerol, and 1 mM ZnSO4) containing 300 units of S1 nuclease (Boehringer Mannheim) was added followed by incubation for 30 min at 30 °C. 100 μl of 5 M ammonium acetate, pH 6.0, containing 20–30 μg of yeast carrier RNA was added, and the samples were phenol/chloroform (1:1)-extracted, chloroform-extracted, ethanol-precipitated, and lyophilized. Samples were then analyzed by electrophoresis in sequencing gels (see below).

For some experiments mitochondrial nucleic acid samples were treated with 0.3 M KOH to remove ribonucleotides attached to DNA termini as described (23). Ribonucleotides were also removed by preincubation of samples for 30 min at 60 °C with 500 μg/ml RNase A.

In all cases, singly 32P end-labeled, single-stranded S1 nuclease probes were prepared by standard techniques from various restriction enzyme digests of Bov 1 plasmid DNA (18, 24). These 5' end-labeled probes were prepared by standard techniques from various restriction ends of transcripts from PL (probes A–C) and PH (probes D and E). These 5' end-labeled restriction fragments of Bov 1. Probes were prepared to map D-loop 5' ends (probes A–C) and the 5' ends of D-loops (probes G–J) as well as PL (probes G–J) and PH (probes J and K) termini as described (23).

**TABLE I**

| Probe | Label | Length | Restriction enzyme |
|-------|-------|--------|--------------------|
|       |       | 5' End | 3' End | 5' End | 3' End |
| S1 nuclease probes |       |        |        |        |        |
| A     | 5'    | 1,479  | 410    | 16,201 |        |
| B     | 5'    | 329    | 627    | 956    | 80     |
| C     | 5'    | 413    | 735    | 1,148  | 188    |
| D     | 5'    | 329    | 956    | 627    | 409    |
| E     | 5'    | 592    | 1,002  | 410    | 455    |
| F     | 5'    | 1,910  | -1,500 | 14,291 | 16,201 |

Primer extension probes

|          |       |        |        |        |        |
| G        | 5'    | 59     | 410    | 469    | 16,201 |
| H        | 5'    | 108    | 627    | 735    | 80     |
| I        | 5'    | 67     | 627    | 694    | 80     |
| J        | 5'    | 30     | 627    | 657    | 80     |
| K        | 5'    | 47     | 1,002  | 956    | 455    |

**RESULTS**

Mapping of D-loop 5' Ends—The 5' termini of bovine D-loops were mapped with S1 nuclease and primer extension assays using probes labeled at the BamHI site (located near the center of the bovine D-loop region; see Table I and Fig. 3). The results of S1 nuclease assays using probe A (see Table I) with mtDNA samples from tissue culture cells and bovine heart are shown in Fig. 2 A (lanes 1–3). Multiple bands corresponding to discrete 5' termini are evident with mtDNA samples from each source. In confluent tissue culture cells and bovine heart, a single, dominant band aligns with nucleotide 158 in the bovine mtDNA sequence (2). (Nucleotide 158 is renumbered nucleotide 705 in Fig. 3 (see legend to Fig. 3).) A different set of bands is observed when mtDNA from growing tissue culture cells is analyzed. Here, the band at nucleotide 705 is virtually absent, and a major band at nucleotide 751 (see Fig. 3) is evident in addition to several larger species.

Because polymorphisms in the D-loop region have been reported between closely related individuals (26), a band in an S1 nuclease assay could represent a true terminus or a major sequence heterogeneity between the probe DNA and the target DNA. Therefore, primer extension assays were performed using a primer extension probe (probe G) labeled at the same site as the S1 nuclease probe (probe A) (Fig. 1A, lanes 4–6). For each mtDNA sample there is good correspondence between the termini identified by S1 nuclease and primer extension analysis, indicating that the bands identified represent true termini.

DNA Sequencing Gels—For most experiments 6, 10, or 15% polyacrylamide (Bio-Rad), 8 M urea (Boehringer Mannheim) sequencing gels were used to analyze S1 nuclease and primer extension experiments. Gels were prepared as described (24). G and A+G sequencing reactions were performed on 5' or 3' singly end-labeled double-stranded DNA restriction fragments of Bov 1 as described (24). These sequencing reactions were used as size markers in most experiments. Before electrophoresis, samples were dissolved in 0.8 μl of 80% formamide containing 1 mM Na2EDTA, 10 mM NaOH, 0.1% xylene cyanol, and 0.1% bromphenol blue. Samples were heated to 90 °C for 1 min before loading onto prewarmed sequencing gels.

2 The differences observed in the D-loop 5' termini of growing and nongrowing cells will be presented more fully in the following paper (25).

3 Preparations of mtDNA from several bovine hearts showed prominent bands at nucleotide 905 in S1 nuclease assays (in the tract of 12 cytosine residues which immediately precede the structural gene for tRNA Phe). This band was much less prominent in primer extension assays, suggesting that it was due to a major sequence heterogeneity (data not shown). Heterogeneity in the length of this cytosine tract has been reported previously (27).
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![Diagram of nucleotide sequence and primer extension assays](image)

**FIG. 2. 6% sequencing gels for D-loop 5' ends.** Maxam and Gilbert sequencing reactions for guanosine (G) and adenosine plus guanosine (A+G) were carried out on a BamHI-HindIII restriction fragment of Bov I plasmid DNA. This fragment was 5' end-labeled at the BamHI site (same site of labeling as probes A and G, see Table 1). In A, lanes 1-3 show S1 nuclease assays using a constant amount of probe A, and lanes 4-6 show reverse transcriptase primer extension assays using a constant amount of probe G (see "Materials and Methods"). Assays in lanes 1 and 4 contained 9.4 ng of mtDNA from growing CCL22 cells; lanes 2 and 5, 9.4 ng of mtDNA from confluent CCL22 tissue culture cells; and lanes 3 and 6, 75 ng of bovine heart mtDNA. The positions of major bands are assigned relative to the sequencing reactions and are numbered in correspondence with Fig. 3. In B, the reactions in lanes 1-4 contained 28 ng of mtDNA from confluent CCL22 cells and those in lanes 5-9 contained 28 ng of mtDNA from growing CCL22 cells. Lanes 1 and 5 are reverse transcriptase primer extension assays using probe G; lanes 2 and 6, Klenow primer extension assays using probe G; lane 7, an S1 nuclease assay using probe A; lanes 3 and 8, S1 nuclease assays using probe A after prior RNase treatment of the samples; and lanes 4 and 9, S1 nuclease assays using probe A after prior treatment of the samples with KOH. Nucleotide assignments are as described for A.

The termini identified in Fig. 2A could represent either RNA or DNA species since S1 nuclease and primer extension assays (using reverse transcriptase) will detect either species. To determine whether RNA was present in the D-loop strands, primer extension assays were repeated using Klenow enzyme (which is unable to use RNA as a template) instead of reverse transcriptase. No major bands were lost or altered in position in the Klenow assays relative to the reverse transcriptase assays, suggesting that RNA-containing species contributed little to the observed signal (compare lane 1 with lane 2 and lane 5 with lane 6 in Fig. 2B). Similarly, pretreatment of mitochondrial nucleic acid samples from growing or nongrowing cells with RNase (Fig. 2B, lanes 3 and 8) or KOH (lanes 4 and 9) did not alter the major termini observed (KOH pretreatment does greatly reduce the signal; see below). One minor band at nucleotide 790 is lost after RNase (and KOH) treatment, suggesting that it arises from a species composed (at least partially) of RNA. This band is not seen in primer extension experiments with reverse transcriptase (Fig. 2B, lane 5, and Fig. 1A, lane 4), however. It likely arises from an RNA species not covalently attached to DNA, with termini such that it can hybridize to an S1 nuclease probe already partially protected by a separate DNA fragment.4 RNA

4 Two separate nucleic acid species can hybridize to a single S1 nuclease probe, thus protecting a greater portion of the probe DNA than would be protected by either species alone (22).
primers with 3’ termini that abut the 5’ ends of D-loop DNA species have been described in both human and mouse systems (28, 29).

Randomly incorporated ribonucleotides have been identified in the mouse mitochondrial genome (30) and tend to be clustered in the D-loop region (31). The sensitivity of D-loop 5’ termini to KOH pretreatment (Fig. 2B) suggests that ribonucleotides are present in the bovine D-loop as well. The signal from growing cells is relatively more susceptible to KOH pretreatment (Fig. 2B) than in nongrowing cells (Fig. 2B, lanes 4 and 9).

Since the probes used in the experiments shown in Fig. 2 have labeled termini located relatively far from the D-loop 5’ ends detected (~300 nucleotides; see Table I and Fig. 3), other probes were constructed to map these termini more precisely. Such an experiment is shown in Fig. 4 and the major and minor termini identified are indicated relative to the DNA sequence in Fig. 3. Mitochondrial nucleic acid samples from growing and confluent cells were either analyzed directly (lanes 1 and 3) or after pretreatment with RNase (lanes 2 and 4). Surprisingly, RNase treatment quantitatively altered the migration of the major band found in confluent cells from nucleotide 705 to nucleotide 703. In growing cells the minor band at nucleotide 705 is similarly shifted in position, although none of the other bands are affected. (The level of resolution with the longer probes employed in Fig. 2 precluded the detection of this size alteration.) These results suggest that at least 2 ribonucleotide residues are attached to the 5’ end of all D-loop species with 5’ termini at nucleotide 705.

Mapping of Light-strand Transcripts—The RNase-sensitive species yielding a band at nucleotide 790 (Fig. 2B, lane 7) appeared to be an RNA transcript from PL. An S1 nuclease probe (probe C) was constructed with its labeled 5’ end located at nucleotide 735 to better characterize this species. When samples from actively growing cells (Fig. 5, lane 6) or from cells approaching confluence (lane 4) were assessed with this probe, a discrete band at nucleotide 790 was evident in addition to the band at nucleotide 772 resulting from a D-loop DNA 5’ end (see Fig. 4). After RNase treatment (lanes 3 and 1) this band is quantitatively removed whereas the band at nucleotide 772 (due to DNA) is unaltered. Samples from confluent cells and from bovine heart showed no detectable band at nucleotide 790 either before or after RNase treatment (Fig. 5, lanes 2, 5, 7, and 8).

The experiments shown in Fig. 4 indicated that a primer extending from nucleotide 627 to nucleotide 657 (probe J) could not prime this RNA species to yield a band at nucleotide 790 (see Fig. 4, lane 3). Therefore, primer extension probes whose 3’ ends extend nearer to nucleotide 790 (probes H and I) were constructed (see Table I). In Fig. 6, the position of the RNA species at nucleotide 790 is marked by an S1 nuclease assay using probe B with mitochondrial nucleic acid from growing cells (lane 6). Lanes 2 and 4 show different exposures of a primer extension assay using probe I (extending to nucleotide 694) with mitochondrial nucleic acid from growing cells. Whereas an intense signal from D-loop DNA 5’ ends is evident in this lane, no band at 790 is seen in the primary exposure (lane 4), although a faint band is present in the overexposure (lane 2). Lanes 9 and 11 show two exposures of a primer extension assay using probe H (extending to nucleotide 735) with mitochondrial nucleic acid from growing cells. In this case a weak band is seen at nucleotide 790 in the primary exposure (lane 9), with a dark band present in the overexposure (lane 11). (The large, dark, diffuse band present

![Fig. 4. 10% sequencing gel for D-loop 5’ ends. All lanes contain reverse transcriptase primer extension assays using a constant amount of probe J. Lanes 1 and 2 contained 28 ng of mtDNA from confluent CCL22 cells, lanes 3 and 4, 28 ng of mtDNA from growing CCL22 cells (different sample from Figs. 1 and 2). The samples in lanes 2 and 4 were pretreated with RNase. Nucleotide size markers (not shown) are as described in the legend to Fig. 2.](image)

![Fig. 5. 15% sequencing gel for D-loop 5’ ends and light-strand transcripts. All lanes contain S1 nuclease assays using a constant amount of probe C. Lanes 1 and 4 contained 28 ng of mtDNA from CCL22 cells which were approaching confluence; lanes 2 and 5, 28 ng of mtDNA from confluent CCL22 cells; lanes 3 and 6, 28 ng of mtDNA from growing CCL22 cells; and lanes 7 and 8, 28 or 56 ng of bovine heart mtDNA, respectively (samples obtained from different animals). Samples for the reactions in lanes 1–3 were pretreated with RNase. Nucleotide size markers (not shown) are as described in the legend to Fig. 2.](image)
The reaction in assays using probe labeled 5' end (see Table I). Note that unreacted probe H is retained reactions in promoter (PI*) have 3' ends which abut D-loop DNA 5' ends shown) are as described in the legend to Fig. 2. CCL22 cells; lane 1 contains no mtDNA. The reactions in lanes 3 and 4 are reverse transcriptase primer extension assays using probe B. The reactions in lanes 5-7 are S1 nuclease assays using probe C. All reactions in lanes 8 and 9 are reverse transcriptase primer extension assays using probe H (lanes 10 and 11 are overexposures of lanes 8 and 9, respectively). All probes used in this experiment had the same labeled 5' end (see Table I). Note that unreacted probe H is retained in this gel at about nucleotide 740. Nucleotide size markers (not shown) are as described in the legend to Fig. 2.

Some of the RNA species with discrete 5' termini at nucleotide 790 have 3' termini located at a position which allows them to be primed by probe H (Fig. 6, lanes 9 and 11) and to a lesser extent by probe I (Fig. 6, lanes 2 and 4), but not at all by probe J (Fig. 4, lane 3). If 15 nucleotides is the minimum sequence length required for hybridization, then a small percentage of this RNA species' 3' termini are located between them to be primed by probe H. These results are consistent with the findings in human and mouse cells that RNA primers from the light-strand cleotide 790 have 3' termini located at a position which allows a lesser extent by probe I (Fig. 6, lanes 2, 4, 6, 9, and 11 contained 19 ng of mtDNA from growing CCL22 cells. The reaction in lane 7 contained no mtDNA. The reactions in lanes 3 and 4 are reverse transcriptase primer extension assays using probe B and to those in lanes 2 and 4 are overexposures of lanes 3 and 4, respectively. The reactions in lanes 5-7 are S1 nuclease assays using probe B. The reactions in lanes 8 and 9 are reverse transcriptase primer extension assays using probe H (lanes 10 and 11 are overexposures of lanes 8 and 9, respectively). All probes used in this experiment had the same labeled 5' end (see Table I). Note that unreacted probe H is retained in this gel at about nucleotide 740. Nucleotide size markers (not shown) are as described in the legend to Fig. 2.

Mapping of D-loop 3' Termini—The 3' termini of D-loops were mapped with S1 nuclease assays using a probe (probe F) 3' end-labeled at the BamHI site near the center of the D-loop region (nucleotide 410; see Fig. 3). In confluent cells (Fig. 7, lanes 1, 2, and 4) a single group of closely spaced bands (6-9 bands) is seen at a position corresponding to nucleotides 173-178 (lanes 1 and 2) or 170-178 (lane 4). The samples in lanes 1 and 2 are from an established bovine epithelial cell line, CCL22, whereas the sample in lane 4 is from a primary culture of bovine endothelial cells, CCL209. An additional group of bands (nucleotides 188-190) is detected in samples from growing epithelial (CCL22) and endothelial (CCL209) cells (Fig. 7, lanes 3 and 5, respectively). The major group of bands seen in CCL22 cells (nucleotides 173-178) broadens from 6 to 9 bands in growing compared to confluent (lanes 1 and 2) cells. Bovine heart mtDNA yielded bands at nucleotides 170-178 and at nucleotide 231 (data not shown).

Since the identification of D-loop 3' termini rests solely on S1 nuclease assays, it is possible that the ends identified represent major sequence heterogeneities between the mtDNA and the S1 nuclease probes, rather than true termini. This possibility seems unlikely to account for all the ends observed for three reasons. First, as can be seen in Fig. 7 (near the top of the gel), a portion of the single-stranded S1 nuclease probe used in each assay is protected over its entire length by genomic mtDNA. This finding mitigates against the possibility of a major sequence polymorphism in the D-loop region. Second, the same termini are observed in two unrelated cell lines, an unlikely finding if a major sequence polymorphism were responsible for the ends observed. Third, different ends are observed in growing and nongrowing cells. The 3' ends of D-loops in bovine brain tissue have recently been mapped by others (16) at more distal positions (see Fig. 3) using a different technique (see "Discussion").

Mapping of Heavy-strand Transcripts—Heavy-strand transcripts were initially mapped using an S1 nuclease probe with its labeled 5' end located within the gene for tRNA Phe (probe C; see Table I). As expected, a strong band is seen at nucleotide 910 in Fig. 8 corresponding to the mature 5' terminus of tRNA Phe. (RNAse treatment of samples prior to hybridization eliminated all of the bands seen in Fig. 8 (data not shown).) In addition, 9 closely spaced bands are present at nucleotides 887-895 (see Fig. 3). No larger bands are seen, although the 3' end of this probe extends beyond the position of most D-loop DNA 5' ends so that much longer transcripts could be detected if present (Table I and Fig. 3). The relative intensity of these individual bands differs in growing and nongrowing cells (see Fig. 8, lanes 1 and 3 (growing cells) and lanes 2 and 4 (nongrowing cells)). The bands at nucleotides 887 and 888 are much more intense in
Growing cells, with large amounts of mitochondrial nucleic acid (10 times greater than that from growing cells) would compete for binding to the S1 probe at higher concentrations of mitochondrial nucleic acid. (If these species are nascent transcripts, they would be at least 100 nucleotides in length (including attached tRNA Phe) and should not displace a species 98 nucleotides in length under the hybridization conditions employed here.)

The ends observed at nucleotides 887–895 could result from small RNA species (nucleotides 910 to 895 or 887) which hybridize to S1 nuclease probes already partially protected by mature tRNA Phe (the same phenomenon encountered with P₇ transcripts; see above). Endonucleolytic processing of P₇ transcripts at the 5' terminus of tRNA Phe could produce such small RNA species. In this case, rare species mapping at nucleotides 887 and 888 should displace smaller species when large amounts of mitochondrial nucleic acid from growing cells are probed (i.e. a species 23 nucleotides in length (position 887; Fig. 9, lane 1) could displace one 15 nucleotides in length (nucleotide 895; Fig. 9, lane 1) under the hybridization conditions employed). The sharp lower limit to the length of these species at position 895 is also explained since shorter species (less than 15 nucleotides in length) would not hybridize efficiently under the conditions employed.

If these arguments are correct, species with 5' termini at nucleotides 887–885 should not be detected in primer extension assays since they are not covalently attached to tRNA Phe. A primer extension assay with mitochondrial nucleic acid from growing cells is shown in lane 2 of Fig. 10. As predicted, no bands are apparent at nucleotides 887–885 although an intense band which corresponds to the mature end of tRNA Phe (nucleotide 910) is present. In addition, other bands are observed which map within the structural gene for tRNA Phe. These bands may represent points in the tRNA's secondary structure that are resistant to unfolding by reverse transcriptase (leading to its blockage and premature termination). Another possibility is that these bands represent alternate initiation sites, although no bands were identified at these positions in S1 nuclease assays (Fig. 10, lanes 1 and

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**Fig. 8. 10% sequencing gel for the 5' ends of heavy-strand transcripts.** The reactions in all lanes are S1 nuclease assays using a constant amount of probe D and 19 ng of mtDNA. The reaction in lane 1 contained mtDNA from growing CCL209 cells; lane 2, confluent CCL209 cells; lane 3, growing CCL22 cells; and lane 4, confluent CCL22 cells. Nucleotide size markers (not shown) are as described in the legend to Fig. 2. Nucleotide 910 corresponds to the mature 5' end of tRNA Phe.

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**Fig. 9. 10% sequencing gel for the 5' ends of heavy-strand transcripts.** The reactions in all lanes are S1 nuclease assays using a constant amount of probe D. The reactions in lanes 1–5 and lanes 6–10 contained varying amount of mtDNA from growing and confluent CCL22 cells, respectively. The reaction in lane 1 contained 19 ng of mtDNA; lane 2, 3.8 ng; lane 3, 380 pg; lane 4, 188 pg; lane 5, 56 pg; lane 6, 56 ng; lane 7, 5.6 ng; lane 8, 1.7 ng; lane 9, 563 pg; and lane 10, 169 pg. Nucleotide size markers (not shown) are as described in the legend in Fig. 2.

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Growing Non Growing

| Lane | Growing | Non Growing |
|------|---------|-------------|
| 1    | 887     | 887-895     |
| 2    | 895     | 895-910     |
| 3    | 910     |             |
| 4    |         |             |

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Why slightly longer species (termini 887 and 888 in growing cells) would compete for binding to the S1 probe at higher concentrations of mitochondrial nucleic acid (If these species are nascent transcripts, they would be at least 100 nucleotides in length (including attached tRNA Phe) and should not displace a species 98 nucleotides in length under the hybridization conditions employed here.)

Growing cells, with large amounts of mitochondrial nucleic acid from growing cells is shown in lane 2 of Fig. 10. As predicted, no bands are apparent at nucleotides 887–885 although an intense band which corresponds to the mature end of tRNA Phe (nucleotide 910) is present. In addition, other bands are observed which map within the structural gene for tRNA Phe. These bands may represent points in the tRNA's secondary structure that are resistant to unfolding by reverse transcriptase (leading to its blockage and premature termination). Another possibility is that these bands represent alternate initiation sites, although no bands were identified at these positions in S1 nuclease assays (Fig. 10, lanes 1 and...
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3. Similar initiation sites in HeLa cells may allow differential expression of tRNA and mRNA genes (32, 33).

DISCUSSION

The identification of the promoters and D-loop termini in bovine cells allows a more comprehensive comparison of this genome with that of other species than has been previously possible. The structural and functional features of the bovine, mouse, and human mitochondrial genomes can be compared in detail. In addition, a direct comparison of sequence elements which subserve homologous functions in the different species is possible. This analysis leads to some inferences about which features of these genomes are important for function and about the relationship of these different mitochondrial genomes to each other.

Functional Organization of D-loop Regions—The D-loop regions of the bovine, human, and mouse mitochondrial genomes are diagramed in Fig. 11A. Here each is drawn to a slightly different scale to align the genes for tRNAs Phe and Pro at either end of the D-loop region. Both PH and PL are positioned in nearly identical relative locations in each genome. The major bovine D-loop 5' ends are located much closer to PL (from which they are apparently primed; see below) than in either the human and mouse genome. This difference is even more pronounced when the D-loop regions are drawn to the same scale and aligned relative to PL as in Fig. 11B. In contrast, the positions of D-loop 3' termini in the human and bovine genomes are similar, whereas in the mouse, many D-loops extend to the gene for tRNA Pro which bounds the D-loop region (see Fig. 11A).

The bovine and mouse D-loop regions are each about 200 nucleotides shorter than the human region. The relative locations of the promoters and D-loop termini suggest that sequences near tRNA Pro have been lost in the mouse genome relative to the human and bovine genomes. In contrast, the bovine D-loop region appears to have lost sequences between PH and OriH relative to the human and mouse genomes. This interpretation is strengthened by comparison of the sequences conserved between the D-loop regions of the human, bovine, and mouse genomes (see Fig. 3 and Ref. 2). The most conserved of these sequences (see Fig. 3, B, C, D, E, F, and J) all reside in the portion of the D-loop region that forms the triplex D-loop structure in each species, and the locations of these sequences support the idea that different regions have
been lost between these species. The conserved sequence blocks (CSB1-3, Ref. 14) identified in the human, mouse, and rat D-loop regions near P1 and OriH do not appear to be conserved in the bovine genome. CSB2 is the only one of the three to show marked homology in the bovine D-loop region. This sequence is located proximal to P9 in the mouse and human genomes but distal to P9 in the bovine genome. All of the other sequences conserved among the mouse, human, and bovine D-loop regions (Fig. 3) are located away from the transcriptional promoters and OriH in the portion of the D-loop region which forms the triplex D-loop structure. The degree of conservation of these sequences suggests that they have a role in controlling mitochondrial gene expression even though they are geographically distant from P9 in the bovine genome. All of the other sequences conserved end observed in confluent cells (nucleotide sequences) suggest that they have a conserved role in controlling mitochondrial gene expression even though they are geographically distant from P9 and P1. Conserved features other than the primary DNA sequence (e.g. secondary structural features) may be important as well.

**D-loop 5' Ends**—It seems likely that the D-loop species identified here are primed by transcripts from P1, as has been described in human and mouse cells (28, 29). The major 5' end observed in confluent cells (nucleotide 705) clearly results from a priming event since this terminus is quantitatively substituted with ribonucleotides. The largest D-loop species observed (nucleotides 762 and 772) may also arise from de novo priming events. However, all of the D-loop 5' termini observed need not result from a priming event. The smaller species could be formed from one or a few larger species by nucleolytic cleavage. Some of the transcripts with 5' termini at P1 clearly have 3' ends located between nucleotides 684 and 772 in positions which would allow them to prime most of the D-loop species observed.

**D-loop 3' Ends**—The D-loop 3' ends identified in this study do not correspond with those previously reported by others (16). Although these differences could result from artifacts of the S1 nuclease technique used here, this possibility seems unlikely for the reasons noted above. Bovine D-loop 3' ends may actually differ in different tissues (brain tissue versus endothelial or epithelial cells in culture). This notion is strengthened by the fact that different D-loop 3' ends are observed in growing and confluent cells (see Fig. 7). Terminus-associated sequences have been described in human (34), mouse (34), bovine (16), and porcine (16) systems. By definition, these short sequences share limited homology within a given species, but they show almost no homology between different species. Sequences similar to the terminus-associated sequences described in bovine brain are not evident at similar positions (about 70 nucleotides upstream; see Ref. 16) relative to the 3' ends described here (although a similar sequence at nucleotide 278 is located 90-100 nucleotides upstream of the observed 3' ends; Fig. 3).

**Light-strand Transcripts**—Although short (presumably priming) RNA transcripts were identified at nucleotide 790, no long (genomic) light-strand transcripts were detected in any experiments. Nascent transcripts from P1 may be rapidly processed endonucleolytically at D-loop priming sites, as has been suggested in mouse cells (35). Indeed, if this model is correct, then a single transcriptional event could both prime DNA replication and produce mRNA and tRNA.

Little sequence homology is present at the bovine light-strand promoter compared to either the mouse or human light-strand promoter (36, 37), and the human promoter consensus sequence (37) is not present in the bovine promoter. However, one feature may be conserved between bovine and human systems. This is a 12-nucleotide sequence located about 10 nucleotides upstream of P9 in each, which is identical in the human and bovine genomes (see Fig. 3). This region (located within the functional human promoter as defined by deletion mapping; see Ref. 36) seems striking both for its degree of homology and for its conserved position relative to each promoter. However, this sequence is entirely lacking in the mouse D-loop region, making the evaluation of its significance difficult. No obvious homology is apparent between the two bovine promoters.

**Heavy-strand Transcripts**—All of the heavy-strand transcripts detected in the present work represent RNA species with 3' ends abutting the 5' terminus of tRNA Phe. As no longer heavy-strand transcripts are detected, it seems likely that the longest of these species (the most abundant in growing cells) represents a true transcription product from the promoter. The position of this site in the D-loop region relative to human and mouse heavy-strand promoters reinforces this inference (36, 37). Whether the shorter termini observed (nucleotides 889-895, Fig. 3) represent degradation products of this nascent transcript (found to accumulate in confluent cells) or alternate transcription start sites (preferentially used in confluent cells) is unknown. Different transcription start sites for RNA and polycistronic mRNAs could allow greater control of gene expression (32, 33).

Processing of nascent transcripts at the 5' end of tRNA Phe must occur very rapidly, as no uncleaved species are detected in primer extension assays (see Fig. 9). These short, apparently stable, RNA species are at least as abundant as tRNA Phe, suggesting that they serve a role in vivo. These species are much more abundant than are D-loop 5' ends or P1 transcripts, as judged by the amounts of mtDNA required to give comparable hybridization signals (see Figs. 4, 7, and 9).

**CONCLUSIONS**

Several important similarities and differences in the structural and functional organization of D-loop regions of the human, bovine, and mouse mitochondrial genomes are apparent. The major similarities are: 1) The promoters for heavy- and light-strand transcription are located in homologous positions in each species (see Fig. 11A). 2) D-loop DNA 5' ends are apparently primed by transcripts from P1 in each species. 3) Regions of tightly conserved nucleotide sequence are found in the portion of the D-loop region that forms the triplex D-loop structure in each species. The major differences are: 1) Very little primary sequence homology is present among the regions containing the promoters and OriH in the different species. 2) Different portions of the D-loop region have apparently been lost (or added) among the bovine, human, and mouse genomes. 3) The pattern of D-loop 5' ends (and to a lesser extent, 3' ends) differs in growing and confluent bovine cells but has not yet been reported in other species.

These findings suggest that the means of control of mitochondrial gene expression may be rather different in the species thus far examined. If this is the case, then comparison of the primary nucleotide sequence at functional sites may be
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fruitsless (as they may have qualitatively different control functions). The characterization of more closely related species (in which the mechanisms for gene control are similar) might clarify this difficulty. Nucleotide sequence differences among closely related species could be interpreted with the view that they conserve a given structure or function rather than alter it (e.g. nucleotide changes that preserve hairpin base-pairing interactions have been described in related rDNA genes).

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