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Template-Directed Arrest of Mammalian Mitochondrial DNA Synthesis

SALLY L. D. MACKAY,[1] PAUL D. OLIVO,[1] PHILIP J. LAIPIS,[2] AND WILLIAM W. HAUSWIRTH[1]*

Department of Immunology and Medical Microbiology[1] and Department of Biochemistry and Molecular Biology,[2] University of Florida College of Medicine, Gainesville, Florida 32610

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Mammalian mitochondrial DNA often contains a short DNA displacement loop at the heavy-strand origin of replication. This short nascent DNA molecule has been used to study site-specific termination of mitochondrial DNA synthesis in human and mouse cells. We examined D-loop strand termination in two distantly related artiodactyls, the pig and the cow. Porcine mitochondrial DNA was unique among mammals in that it contained only a single species of D-loop single-stranded DNA. Its 3′ end mapped to a site 187 nucleotides from the 5′ end of the proline tRNA gene. This site was 21 and 47 nucleotides 5′ to two very similar sequences (5′ ACATATPyATTAT 3′) which are closely related to the human and mouse termination-associated sequences noted by Doda et al. (J. N. Doda, D. T. Wright, and D. A. Clayton, Proc. Nat. Acad. Sci. USA 78:616–6120, 1981). Bovine mitochondrial DNA contained three major D-loop DNA species whose 3′ ends mapped to three different sites. These sites were not found in the porcine sequence. However, the bovine termination sites were located 60 to 64 base pairs 5′ from sequences which were also very similar to the termination-associated sequences present in pigs and other mammals. These results firmly establish the concept that arrest of heavy-strand DNA synthesis is an event determined, at least in part, by template sequence. They also suggest that arrest is determined by sequences which are a considerable physical distance away from the actual termination site.

Mitochondrial DNA (mtDNA) in mammalian cells exists as a covalently closed, circular, supercoiled, double-stranded DNA molecule approximately 16,000 base pairs in length (13). Each strand is replicated unidirectionally from a separate origin. Although synthesis of daughter heavy (H) strands begins at discrete genome locations, the precise nucleotide sequence requirements for H-strand initiation have not yet been determined and appear to vary considerably among mammals (10). After initiation, most newly initiated H strands terminate <700 nucleotides downstream. This leads to a partial relaxation of parental supercoiled molecules because the short, newly synthesized H strand, variously termed D-loop strand (11), 7S DNA (4–7, 12, 15, 19), or DH-DNA (7–9), remains associated with the template, thus creating a triple-stranded structure known as a displacement loop (D-loop) molecule. The major form of mammalian mtDNA isolated from cells is this triple-stranded, partially replicated D-loop molecule (10).

Previous studies mapping the 3′ ends of D-loop strands suggested that a portion of the template sequence may be involved in the arrest of H-strand synthesis within the D-loop region. D-loop molecules isolated from mouse L cells contain four major D-loop strands with four distinct 3′ ends (11), while those of human HeLa cells contain three major D-loop strands but only one major 3′ end (12). The existence of a conserved nucleotide sequence approximately 30 to 60 nucleotides from each 3′ end prompted Doda et al. (11) to propose that this sequence may play a role in determining the site of D-loop strand arrest. To test this deduction and to extend our understanding of the regulation of D-loop synthesis during DNA replication in mitochondria, we have determined the precise sites of termination of the D-loop strands in two species (cow, *Bos taurus*; and pig, *Sus scrofa*) from the order *Artiodactyla*. These animals are more closely related than primates are to rodents, but they are still sufficiently divergent (about 40 million years) that significant sequence changes are expected to have occurred within the D-loop region (22).

MATERIALS AND METHODS

Isolation of mtDNA. Mitochondria were isolated from brain tissue by differential centrifugation, and mtDNA was purified as previously described (17). The large *Eco*RI fragments, which contain the D-loop regions in both bovine and porcine mtDNA, were cloned into pACYC184 (S. M. Tanhauser, Ph.D. thesis, University of Florida, Gainesville, 1985). To facilitate DNA sequencing, a pig mtDNA *Bam*HI fragment containing the 3′ end of the D-loop was cloned into pBR322.

End labeling of D-loop strands. Approximately 5 μg of closed circular mtDNA was heated to 90°C for 1 min and then quick-chilled on ice to release D-loop strands. This DNA, which contains the only free 3′ and 5′ ends, was then directly labeled with either terminal deoxynucleotidyltransferase and dideoxy [α-^{32}P]ATP as described by Tu and Cohen (21) or T_4 polynucleotide kinase and [γ-^{32}P]ATP (18).

Annealing and restriction of D-loop strands. End-labeled D-loop strands were electrophoresed on 8.3 M urea–4% polyacrylamide gels and eluted from the gel. *Eco*RI digestion of cloned mtDNA from both pig and cow yielded fragments for each species which contained the entire D-loop region. Approximately 5 μg each of these fragments was isolated from agarose gels by electrophoresis onto DEAE membranes (NA-45; Schleicher & Schuell), using conditions suggested by the supplier, and then coprecipitated with about 0.1 μg of the complementary D-loop strand. These mixtures were then denatured at 90°C for 10 min in 50% formamide and reannealed immediately in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 50%

* Corresponding author.

TABLE 1. Sizing of artiodactyl D-loop strands[a]

Species	No. of bands	Size (bases)
Pig	1	485
Camel	2	450
		405
Goat	4	590
		550
		520
		480
Cow	3	520
		480
		440

[a] The D-loop strands from each artiodactyl species were 3′ end labeled, and the lengths and number of strands were determined by electrophoresis relative to an end-labeled *Hpa*II digest of pBR322 DNA.

formamide for 3 h at 50°C. Approximately 50% of each labeled D-loop strand annealed to its complementary template strand. The bovine D-loop strand and its hybridized complement were then digested with *Rsa*I. *Sau*3AI or *Hin*cII was used to digest the porcine D-loop strand-template hybrid.

Nucleotide sequence analysis of D-loop strands. The chemical procedure of Maxam and Gilbert (18) was utilized for the sequence determination of the porcine D-loop region and for the sequencing ladders used in mapping termination sites. Nucleotide sequence analysis was carried out with the NUCALN program of Wilbur and Lipman (24) (K-tuple size of 3, window size of 20, and gap penalty of 7) running on a VAX 11/750 minicomputer. Termination-associated sequences (TAS) were identified with the "SEQAID" site search program available from D. J. Roufa, Molecular Genetics Program, Kansas State University, Manhattan. An initial set of generalized search sequences, of the form $ACAT_{1-2}\ AN_{1-3}\ APyN_{1-3}\ AT$, was used to search available mammalian mtDNA sequences. "N" is A or pyrimidine in the first instance and must contain at least one pyrimidine in the second. All know TAS are found by this procedure in either mouse or human mtDNA as well as are a small number of additional sequences (three in human and four in mouse). Fifteen such sequences are found in bovine mtDNA; six are found in the D-loop. Two acceptable sequences are found in the pig mtDNA D-loop (positions 293 and 319); no other matches to the search sequence are found in the 3,439 sequenced bases of pig mtDNA. If these mammalian sequences are further selected according to the more restrictive consensus indicated in Fig. 4, the set of TAS is reduced to only those shown in Fig. 4 and one additional sequence each for human, mouse, and cow. The extra sequences for human and cow are unlikely TAS because they are approximately 16 and 8 kilobase pairs 3′ to all known termination sites, respectively. The extra mouse sequence, at position 15,641, does not align with any previously determined mouse D-loop strand 3′ end (11). We note that it is the shortest of all sequences agreeing with the TAS consensus in Fig. 4 and may be ineffective for that reason.

RESULTS

To determine which animal species would be most informative in understanding D-loop structure, we first surveyed four artiodactyls for the number and approximate size of their D-loop strands. Because this DNA contains the only discrete ends available in mtDNA prepared from freshly isolated mitochondria, we were able to directly label the 3′ ends of the D-loop strands in mtDNA from brain tissue of camel, cow, goat, and pig. The labeled strands were then sized by gel electrophoresis. The results (Table 1) indicate that porcine mtDNA contains a single D-loop strand, while other artiodactyl species have multiple strands of different lengths. Accurate sizes for the porcine and bovine D-loop strands were determined by both 3′ end labeling (Fig. 1) and 5′ end labeling (data not shown). By either technique a single porcine D-loop strand and three bovine strands were the major species present in each mitochondrion. These two artiodactyl species were selected for further analysis. The pig appears to be unique among the mammals thus far analyzed in that a single D-loop strand predominates. Occasionally the porcine D-loop strand appeared as an artifactual doublet (Fig. 1) due to alternative secondary structures (unpublished observations). The very minor 3′ ends occasionally seen in sizing gels (e.g., Fig. 3B) are not derived preferentially from either member of this doublet.

DNA sequence determination. The entire bovine mtDNA sequence had been previously determined (3). Physical and genetic mapping of pig mtDNA located the mitochondrial

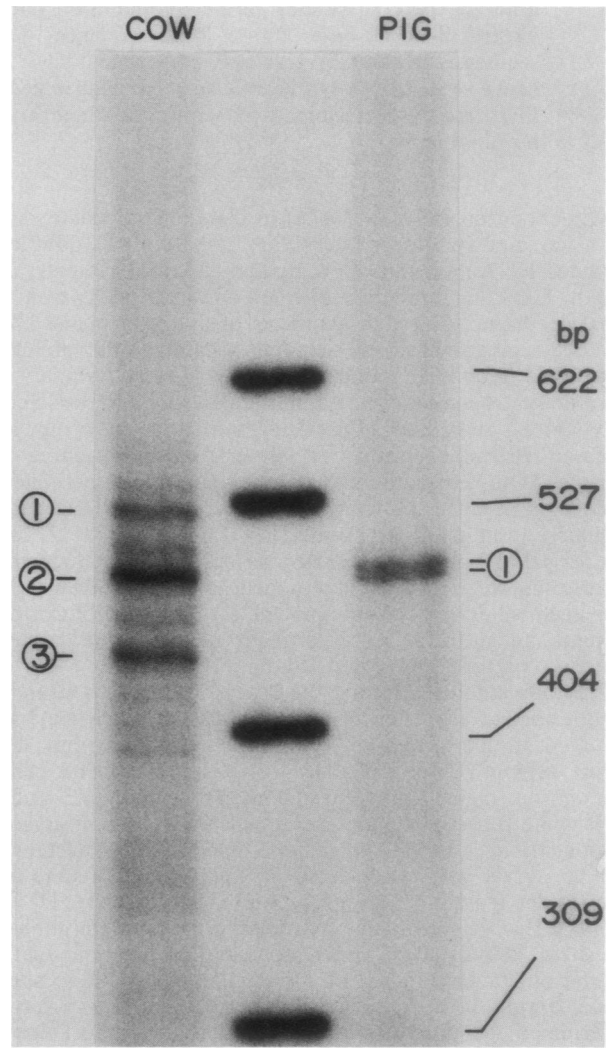


FIG. 1. Autoradiograph of a preparative 8.3 M urea–4% polyacrylamide gel of cow and pig D-loop strands labeled at their 3′ ends. The middle lane contains end-labeled, *Hpa*II-digested pBR322 DNA. bp, Base pairs.

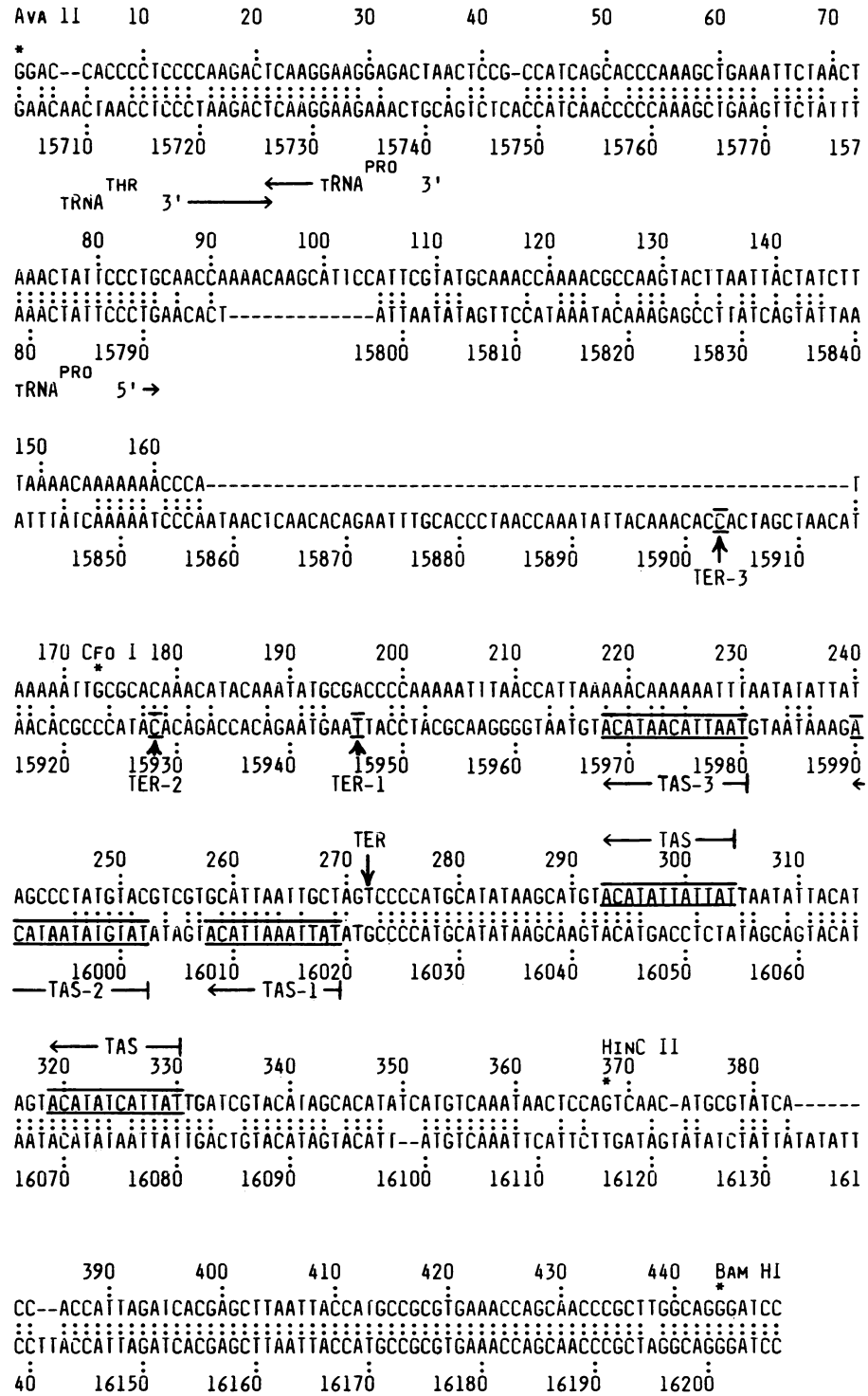
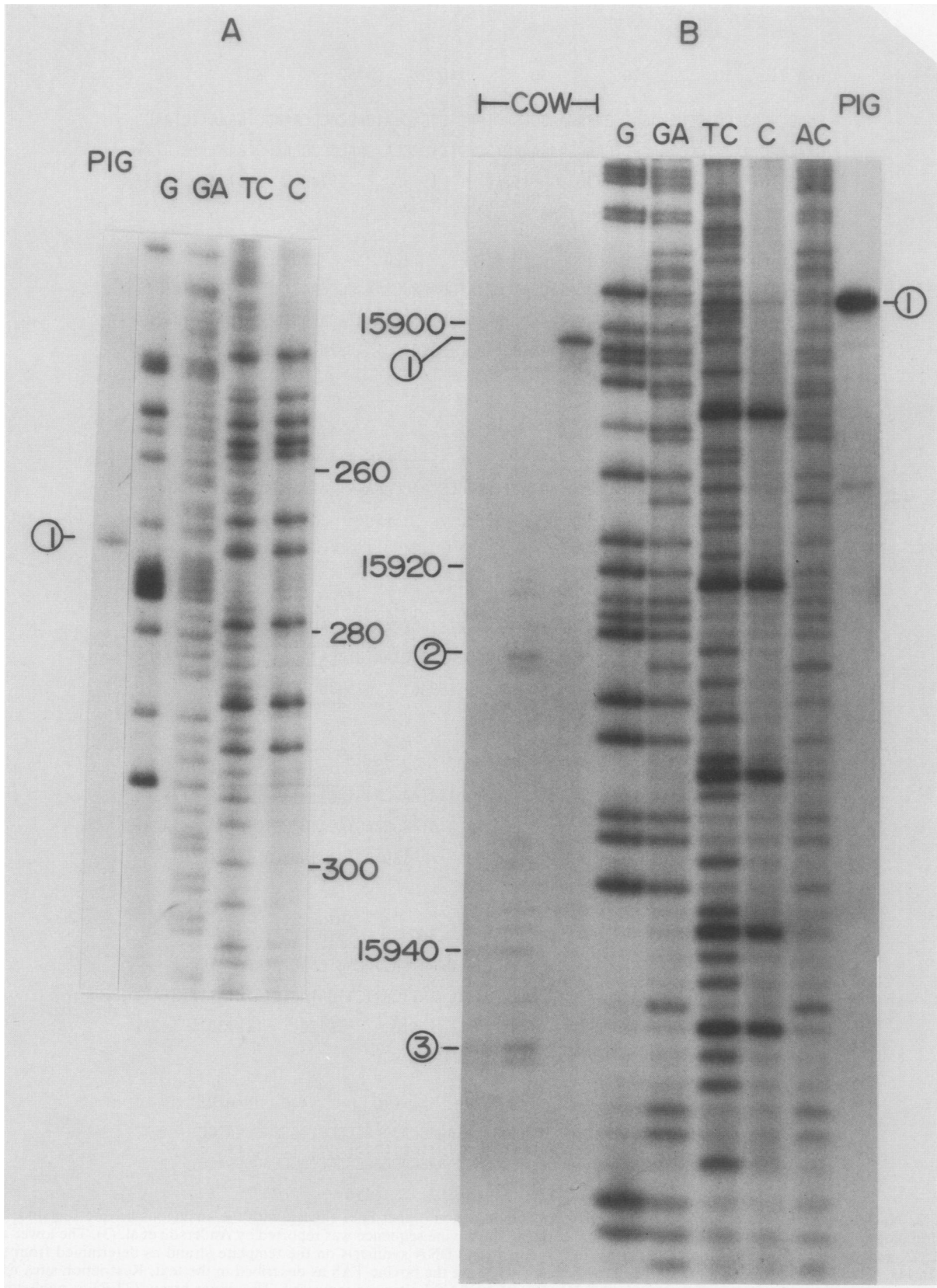


FIG. 2. Nucleotide sequence of the 3′ end of the porcine D-loop region (upper line) aligned with the analogous bovine sequence (lower line). Homology is indicated by dotted lines between nucleotides. The bovine sequence was reported by Anderson et al. (3). The lower arrows (TER-1, -2, and -3) mark the termination sites of bovine D-loop strand DNA synthesis on the template strand as determined from the gel shown in Fig. 3B. The boxed sequences (TAS-1, TAS-2, and TAS-3) are the bovine TAS as described in the text. Restriction sites used for determining the porcine sequence include *Ava*II, *Bam*HI, *Hinc*II, and *Cfo*I sites, as indicated. The upper arrow (TER) at nucleotide 272 denotes the single porcine termination site (see also Fig. 3A and B). The boxed porcine sequences are the TAS as described in the text.


A
PIG
G GA TC C
-260
-280
-300
B
COW
PIG
G GA TC C AC
15900
15920
15940
1
2
3

SPECIES	TAS
MAN	ACAT AA AA A CCC AAT
MOUSE	ACATT AA A CT AAT
	ACAT AA A TC AAT
	ACATT AA A TC AAT
	ACATT AA A CT AAT
COW	ACATT AA A TT AT
	ACAT AA T A TGT AT
	ACAT AA C A TT AAT
PIG	ACAT A TT A TT AT
	ACAT A TC A TT AT
CONSENSUS	ACAT(T) A(A) (AA,Py,PyPy) A Py(N)Py (A)AT

FIG. 4. TAS from all mammalian species thus far analyzed. Below, a consensus based on these TAS and a computer search for mammalian mtDNA sequences is shown (see Materials and Methods).

rRNA genes in the largest of three *Eco*RI fragments, due to the presence of two highly conserved *Sst*II sites (Tanhauser, Ph.D. thesis, 1985). This fragment was cloned into pACYC184 and an internal *Bam*HI fragment was subcloned into pBR322. Mapping data and Southern blotting, using end-labeled porcine D-loop strands as probe, localized the 3′ end of the D-loop strand in this subclone which was then used for sequence determination.

Figure 2 is a comparison of bovine and porcine D-loop sequences. Sequences were aligned beginning with the *Bam*HI site at nucleotide 16,202 in the cow. The highly conserved sequence block in which it occurs is present in all mammals (2–4) and the *Bam*HI site itself is conserved in all artiodactyl species examined. This conserved domain extends for 63 nucleotides toward the proline tRNA gene. Beyond that point, several short, conserved regions as well as some sporadic homology are evident for another 307 nucleotides to a point just before the 5′ ends of the proline tRNA gene. Alternative sequence alignments are possible between bovine and porcine sequences in this region. In addition, there are several apparent deletions, including 1 of the 57 nucleotides in pig relative to the cow sequence. The best overall homology for this region is only about 40%, even if these deletions are ignored. It is within this poorly conserved domain that H-strand DNA synthesis terminates to create D-loop molecules. Therefore, an accurate mapping of termination sites within this region should help to assess the role, if any, played by conserved nucleotide sequences in H-strand arrest.

Location of porcine and bovine D-loop strand 3′ ends. The 3′ end-labeld porcine D-loop strand shown in Fig. 1 was isolated from the gel, reannealed to its complementary strand, cleaved with *Hin*cII at nucleotide 371, and electrophoresed in a sequencing gel. An adjacent lane contained a sequencing ladder of the complementary template strand which was 3′ end labeled at the same *Hin*cII site. The position of the cleaved D-loop strand fragment in relation to the sequence ladder therefore locates the exact 3′ end of the porcine D-loop (Fig. 3A). To confirm this result, the same fragment (digested with *Sau*3A1) was electrophoresed alongside a similar bovine mtDNA sequencing ladder (Fig. 3B). The porcine D-loop primarily terminates at a single position, nucleotide 272 in the porcine sequence. The three distinct D-loop strands present in bovine mtDNA (Fig. 1) were also individually purified from the gel and analyzed as above. Three major termination sites, occurring at nucleotides 15903, 15928, and 15946 in the bovine sequence, are present. A number of less frequently used sites are also evident, similar to those seen in other mammalian systems (6, 12).

The location of the 3′ ends of both porcine and bovine D-loop strands are indicated in Fig. 2. It is clear that the position of the porcine 3′ end does not correspond in position or sequence to any bovine site. Also, the distance between the 3′ end and the conserved *Bam*HI site is not equivalent to any bovine site. Significantly, however, all termination sites are located a short distance downstream from blocks of sequences which show homology both within and between cow and pig. These related sequences (indicated in Fig. 2) share strong similarities with the termination-associated sites previously reported (12) (Fig. 4). Finally, we note that the distances between the 3′ termination sites and the *Bam*HI restriction enzyme site in the cow account for most of the size differences among full-length D-loop strands seen in Fig. 1. This suggests that in the cow, as well as in the pig, the 5′ end of H strands may initiate at one or a few, closely spaced sites.

DISCUSSION

We have precisely located the 3′ termini for the nascent D-loop H strands in both the cow and the pig. No inter- or intraspecific sequence homology is apparent at any of these sites nor is there any obvious spatial relationship. The cow has three major and a number of minor termination sites and thus resembles mouse mtDNA, which also has multiple termination sites. No homology to the mouse D-loop termination sites is obvious. However, as with mouse and human mtDNA, blocks of nucleotides exist near each termination site which resemble the previously noted TAS (11, 12). Four candidate TAS can be found in the bovine sequence, three of which are positioned 60 to 64 nucleotides 5′, respectively, to each D-loop strand 3′ end (Fig. 2 and 4; see Materials and Methods). This confirms the general arrangement of TAS relative to 3′ ends. However, it is not strong proof for the involvement of TAS in actually specifying termination of DNA synthesis because other TAS-like sequences exist in this region (see Materials and Methods) and do not appear to specify major termination sites at an appropriate distance. Finally, we note that the indicated three sites of cow D-loop strand termination are actually three groups of ends differing in length within each family by single nucleotides. This could reflect imprecise termination or nucleolytic degradation after

FIG. 3. Structural mapping of the 3′ ends of porcine (A and B) and bovine (B) D-loop strands. (A) The single species of the porcine D-loop strand (see Fig. 1) was isolated, hybridized, digested with *Hin*cII, and electrophoresed in parallel with a DNA sequence ladder generated by chemical cleavage of the analogous porcine mtDNA restriction fragment also labeled at the *Hin*cII site. This analysis, as described by Doda et al. (11), allows determination of the 3′ end of the D-loop strand by alignment with the corresponding position in the DNA sequence lanes. The exact 3′ ends of the D-loop strands actually map two nucleotides lower on the sequence ladder because the 3′ end-labeling procedure adds an extra nucleotide and the true length of a fragment in a sequencing ladder is one nucleotide less (18). (B) The three major bovine D-loop strands shown in Fig. 1 were individually isolated, hybridized, digested with *Rsa*I, and electrophoresed in parallel with the corresponding bovine mtDNA sequence. A similarity prepared 3′ end-labeled porcine D-loop strand, digested with *Sau*3A1, was also electrophoresed. The three major bovine termination sites, 1, 2, and 3, and the single porcine site are indicated. G, GA, TC, C, and AC refer to each base-specific sequencing reaction.

termination or both. Clearly, however, three modal positions exist which are most likely related to three termination events.

A more rigorous test of the hypothesis that TAS govern the arrest of H-strand synthesis is provided by the porcine D-loop strand. Porcine mtDNA has only one D-loop strand. This represents the simplest picture of initiation and termination of DNA synthesis in mammalian mitochondria. A TAS sequence should therefore occur at an appropriate location relative to the D-loop strand 3′ end and nowhere else in the mitochondrial genome. Examination of the known porcine mtDNA sequences, including the light strand origin of replication, seven tRNA genes, the entire URF-1 gene, 20% of the URF-2 gene, and the gene for the small rRNA (Tanhauser, Ph.D. thesis, 1985; S. L. D. MacKay, unpublished data), as well as the sequences reported here (encompassing 3,439 nucleotides in total), found only the two TAS shown in Fig. 2. They are almost identical, 12-nucleotide segments positioned 14 nucleotides apart on the L strand and begin 21 and 47 nucleotides 3′ to the D-loop strand termination site. Clearly, this suggests that the concept of sequence-associated termination is valid.

The sequences of all known mammalian TAS are shown in Fig. 4. They suggest that, although there is variation in the central portion of TAS among different species, all begin with the sequence ACAT at their 5′ end, are quite A+T rich, and end with a 3′ A−T sequence. It is possible that differences in the nuclear-encoded gene products which may be involved in mtDNA sequence recognition (1, 20, 23) could alter the exact sequence somewhat from species to species. However, that TAS and position are conserved over one hundred million years within the most variable region of the rapidly evolving mammalian mitochondrial genome strongly suggests a function related to arrest of H-strand synthesis.

Although the function of replication arrest is unclear in any system, for mammalian mitochondria a need for such pausing is apparent (10). Initiation of H-strand synthesis occurs throughout the cell cycle (5). Therefore, to maintain a relatively constant intracellular mitochondrial genome pool, termination of H-strand DNA synthesis may serve a regulatory function in controlling mtDNA copy number. Also, if most mitochondrial genomes are to remain transcriptionally active, such arrest should occur before a nascent D-loop strand encounters the first downstream gene, proline tRNA, about 700 nucleotides from mammalian origins of H-strand synthesis (2–4, 16; unpublished data). Recent data suggest that, although the promoter for L-strand transcription lies 5′ to the origin of H-strand DNA synthesis (8, 9, 14), L-strand transcription simultaneously serves both to prime H-strand DNA synthesis (D-loop strand synthesis) and to synthesize the mRNA for downstream L-strand genes (8, 9). Therefore, as long as RNA-primed H-strand DNA synthesis terminates before entering any gene, all L- and H-strand genes should be available for transcription. Thus, termination of H-strand synthesis mediated by TAS may not only regulate mitochondrial genome copy number, but also indirectly control transcriptional activity. Our results considerably strengthen the concept that this termination is signalled by a specific, conserved nucleotide sequence.

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