Mapping light strand transcripts near the origin of replication of *Xenopus laevis* mitochondrial DNA

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ABSTRACT

Transcription of the light strand of *Xenopus laevis* mitochondrial DNA initiates at two promoters located approximately 350 to 450 nucleotides upstream from the 5′ ends of major D-loop DNA strands. Small RNAs within this region have been mapped by blot hybridization, primer extension and S1 nuclease protection methods. The results reveal that the large majority of RNAs within this region have 3′ termini located at a sequence element, designated CSB 2, that is conserved in sequence and position in *Xenopus*, mouse, rat and human mtDNA. However, the *X. laevis* CSB 2 appears to be a site of RNA processing only, since RNA-to-DNA transitions are not detectable at this site. RNAs containing sequences downstream of CSB 2 are extremely rare. A significant fraction of these RNAs are processed by cleavage at a site just upstream of the most predominant 5′ ends of D-loop DNAs. We suggest that RNA processing at this site may play a role in priming mtDNA replication.

INTRODUCTION

The mechanism for replication of mitochondrial DNA (mtDNA) in vertebrates has been studied most extensively in cultured human and mouse cell lines (reviewed in 15). This model suggests that there are two separate origins for continuous synthesis of the two strands of the mtDNA genome, designated the heavy (H-) and light (L-) strands. Replication proceeds by an asymmetric process in which unidirectional initiation from the H-strand origin produces extensive displacement of the parental H-strand. L-strand replication initiates only after the H-strand replication fork has passed the L-strand origin located 10 to 11 kb away from the H-strand origin. A substantial fraction of mtDNA molecules contain a partially replicated segment at the H-strand origin, termed the displacement loop, or D-loop, in which H-strand replication is arrested at specific sites. At least in the case of human mtDNA, these D-loop DNA strands have the same 5′ termini as more extensively replicated molecules (21). D-loop DNA strands have often been considered to represent potential primers for a full round of replication, although this has not been directly demonstrated.

Clues to the detailed mechanism of initiation of H-strand DNA replication have been sought through the analysis of the 5′ ends of nascent daughter strands. Short RNA extensions were first observed in mouse D-loop mtDNA by Gillum and Clayton (17). These RNA-to-DNA transitions were initially thought to reflect the action of a primase. More recent experiments have suggested that transcription by the mitochondrial RNA polymerase actually primes DNA synthesis for both mouse and human mtDNA. Clayton and coworkers have presented evidence for RNA:DNA transitions at highly conserved sequences, designated CSB 1, 2 and 3, in the vicinity of the major D-loop DNA termini (9, 10). The RNA processing event at CSB 2 has been particularly well studied (11, 12, 22).

We have been interested in determining whether this proposed mechanism for priming of leading strand replication applies to mtDNA of the amphibian, *Xenopus laevis*. The *Xenopus* system is of particular interest since mtDNA replication is under developmental control during oocyte development and early embryogenesis. MtDNA is overreplicated in early oocyte development such that a single oocyte contains sufficient mtDNA to endow 50,000 to 100,000 somatic cells of a swimming tadpole (7, 23). MtDNA replication is essentially inactive in early embryonic development (14). It is possible that some of this regulation of mtDNA replication is effected at the level of initiation of H-strand replication. Previous studies have shown that a substantial fraction of *Xenopus* mtDNA has displacement loops (18) and that the D-loops frequency appears to be developmentally controlled under some circumstances (1, 8). We have mapped the ends of the major D-loop DNAs in *Xenopus* (6) and have identified two light strand promoters positioned so as to direct transcription through CSB 3, CSB 2 and the 5′ end of the D-loop DNAs (3). Thus, these landmarks occur in the H-strand origin region of *X. laevis* mtDNA in the same order in which they are found in mouse and human mtDNA. In this paper, we present results of RNA and DNA mapping experiments designed to detect RNA and DNA ends in the general H-strand origin region. The results permit a definitive mapping of abundant small RNAs in this region. However, these results do not suggest a role for RNA processing at CSB 2 or CSB 3 in the generation of 3′ OH termini to serve as RNA primers for the most abundant D-loop DNA strands.

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MATERIALS AND METHODS

Materials

Adult female *Xenopus laevis* were purchased from Xenopus I (Ann Arbor, MI); Avian myeloblastosis virus reverse transcriptase was purchased from Life Sciences, Inc. (St. Petersburg, FL); nuclease S1 was from Boehringer Mannheim (Indianapolis, IN); the bluescript plasmid, pBS, and T7 and T3 RNA polymerases were purchased from Stratagene (La Jolla, CA); restriction enzymes and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA); T4 DNA ligase was from Bethesda Research Laboratories (Gaithersburg, MD); bacterial alkaline phosphatase was obtained from Cooper Biomedical (Malvern, PA); phagemid pTZ19U was obtained from BioRad Laboratories (Rockville Centre, NY); radionucleotides were purchased from ICN Radiochemicals (Irvine, CA); Nytran membrane was purchased from Schleicher and Schuell (Keene, NH). Oligonucleotides were synthesized by solid state phosphoramidite chemistry and were obtained either from a departmental facility or from Genetic Designs, Inc. (Houston, TX). The oligonucleotides A and B used as hybridization probes were purified by HPLC, labeled by phosphorylation with γ-^{32}P-ATP and repurified by gel electrophoresis. All other chemicals were of the highest quality grade commercially available.

Preparation of mitochondrial nucleic acids

Mitochondrial nucleic acids were obtained from mitochondria prepared from homogenates of *X. laevis* ovaries as described (3).

Recombinant DNAs and hybridization probes

The experiments presented in this paper employ probes derived from a cloned segment of *X. laevis* mtDNA extending from a Sau 3A site at residue 461 to an Eco RI site at residue 1246 of the sequence reported by Cairns and Bogenhagen (6). To generate the 3′ labeled probe for the S1 nuclease mapping experiment in Figure 4, this segment of mtDNA was transferred into the phagemid pTZ19U and oligonucleotide directed mutagenesis was used to insert a G residue at position 804 to create a Hind III restriction cleavage site using the method of Kunkel (20). The hybridization probe was prepared by cutting the DNA with Hind III, adding a single A residue by incubation with AMV reverse transcriptase and α-^{32}P-dATP, and recutting the DNA with Sau 3A. The resulting labeled 344 nucleotide fragment was purified by electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

Two short segments of mtDNA were cloned into the vector pBS- to permit the production of short RNA hybridization probes. Clone A contained mtDNA residues 541 to 673 inserted in an orientation such that transcription of the Eco RI digested DNA with T3 RNA polymerase would yield a 197 nucleotide RNA homologous to mtDNA L-strand transcripts. Transcription of the Hind III digested DNA with T7 RNA polymerase yields a 193 nucleotide RNA with the same sense as the mtDNA L-strand. Clone B contained two tandem copies of mtDNA residues 704 to 809 inserted in an orientation such that transcription of the Eco RI digested DNA with T3 RNA polymerase would yield a 276 nucleotide RNA homologous to mtDNA L-strand transcripts. Transcription of the Hind III digested DNA with T7 RNA polymerase yields a 272 nucleotide RNA with the same sense as the mtDNA L-strand.

Three oligonucleotide probes used in this study were designated oligo A, containing mtDNA residues 635 to 649 (CCACCGGGGTTGAGA), oligo B, containing mtDNA residues 809 to 829 (GCTGTATAGAAGACATTTCTC), and oligo D, containing residues 456 to 472 within the D-loop (TCTGGGATCTCAATAGC).

Table I

Approx. RNA size	Hybridization to oligonucleotide	5′ end	3′ end
320	B, A	905 (LSP 2)	585
300	B	975 (LSP 1)	682 (CSB 2)
230	B	905 (LSP 2)	682 (CSB 2)
180	B	864	682 (CSB 2)

Northern blot hybridization

Nucleic acid samples were precipitated with ethanol, resuspended in RNA loading buffer containing 98% formamide, 0.5% sodium dodecyl sulfate, 25 mM EDTA, 0.02% xylene cyanol and 0.02% bromophenol blue, heated at 70°C for 10 min and subjected to electrophoresis on a 6% polyacrylamide gel containing 8 M urea. Following electrophoresis, the nucleic acids were transferred to Nytran membranes by electroblotting for 1 hr at 110 Volts with a BioRad Transblot electroblotting apparatus in 10 mM Tris-borate buffer containing 0.2 mM EDTA. The prehybridization and hybridization solutions were identical except for the inclusion of a labeled probe in the hybridization. The buffer used was 6× SSC containing 50 mM NaPi, 5× Denhardt's reagent (the 5× formulation includes 0.1% (w/v) each of bovine serum albumin, ficoll and polyvinylpyrrolidone), 0.1 mg/ml calf thymus DNA and 0.1% sodium dodecyl sulfate. When the hybridization was performed with an oligonucleotide probe, the filter was incubated at 37°C overnight. Filters were washed as described by Wood et al. (24). When the hybridization was performed with an RNA probe, the prehybridization and hybridization buffers included 50% formamide and the incubations were performed at 37°C.

S1 nuclease mapping and primer extension

These mapping experiments were conducted as described (3, 25).

RESULTS

Organization of the origin region

The major features of the origin region of *X. laevis* mtDNA are illustrated in Figure 1. This map is drawn with the mtDNA L-strand extending in 5′ to 3′ orientation from left to right. The 5′ portions of two major D-loop DNA species with 5′ termini at residues 519 ± 1 and 549 ± 1 (6) are shown at the left of the diagram. The mtDNA H-strand would extend in the 5′ to 3′ orientation from right to left. The H-strand is generally diagrammed as extending in a clockwise direction in standard maps of circular mtDNA genomes (15). Figure 1 also shows the locations of matches to two highly conserved sequences in vertebrate mtDNA, CSB 2 and CSB 3. The D-loop region is bounded at the right of Figure 1 by the tRNA^{Phe} gene. The D-loop region contains two clusters of bidirectional promoters separated by approximately 70 nucleotides and located 30 and 100 nucleotides upstream of the tRNA^{Phe} gene. The identification of initiation sites and required nucleotide sequences for these promoters have been published (2, 3, 4). The heavy strand promoters (HSP1 and HSP 2) are situated to permit

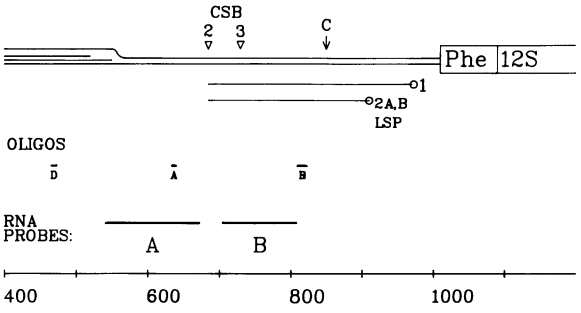

Fig. 1. Map of the *X. laevis* mtDNA origin region.

transcription first through the gene for $tRNA^{Phe}$, then through the rRNAs, and, eventually, through most other tRNA genes and 12 of the 13 mRNAs in the mitochondrial genome. The transcripts from the overlapping light strand promoters (LSP 1 and LSP 2A, 2B) employ the mtDNA L-strand as template and extend into the D-loop region. In the promoter 2 region there are two closely juxtaposed promoters LSP 2A and 2B. Of these two promoters, the downstream promoter, LSP 2B appears to be more active *in vivo*. Only the light strand promoters are diagrammed in Figure 1. The two RNAs diagrammed in Figure 1 were first identified as nascent transcripts containing triphosphate termini capable of being 'capped' *in vitro* with guanylyl transferase and GTP (3). The sizes of these RNAs, approximately 300 and 230 nucleotides, are consistent with initiation at LSP 1 and LSP 2B, respectively, with a common 3′ end near CSB 2. The 3′ termini of these RNAs are mapped in greater detail below.

Abundant small RNAs map in the origin region

Since our preliminary experiments suggested that a substantial fraction of light strand transcripts appeared to be processed at CSB 2, we subcloned short sequences of mtDNA on either side of CSB 2 in the vector pBS- to permit synthesis of short RNA hybridization probes. These two riboprobes are diagrammed in Figure 1. Saturation hybridization experiments performed with these two riboprobes indicated that RNAs upstream of CSB 2 were approximately 5% as abundant as 12 S rRNA, while RNAs downstream of CSB 2 were only 0.5% as abundant as 12 S rRNA (M. Morvillo, unpublished observations). The same RNA probes were employed in Northern blot hybridizations to identify RNAs containing sequences either upstream or downstream of CSB 2. Blot hybridizations were also conducted with synthetic oligonucleotides A and B (Fig. 1) as more precise hybridization probes.

The results of hybridization with RNA probes and oligonucleotide probes are shown in Figures 2 and 3, respectively. These two hybridization strategies revealed the same RNA species. We have included the results with RNA probes (Fig. 2) to show that the use of oligonucleotide probes (Fig. 3) did not exclude any abundant RNAs. The 'upstream' RNA probe B and oligonucleotide B hybridized to three discrete short RNAs 300, 230 and 180 nucleotides in length (Figs. 2B and 3B). These short RNAs appear to represent the abundant RNA sequences detected by saturation hybridization experiments discussed above. The larger two RNAs are likely to have 5′ ends at LSP 1 and LSP 2, respectively, since similar RNAs were previously detected following *in vitro* capping of mtRNA (3). Additional experiments to map the ends of these RNAs and of the shorter 180 nucleotide RNA are presented below. The 'downstream' RNA probe A and

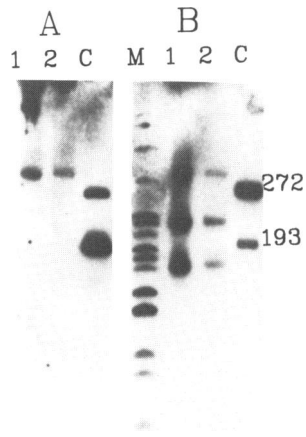

Fig. 2. Identification of origin region transcripts with short riboprobes mapping on either side of CSB 2. 10 μg samples of two different preparations of total mitochondrial nucleic acids were subjected to electrophoresis in lanes 1 and 2 of a denaturing polyacrylamide gel containing 8 M urea along with a hybridization control sample in lanes C. These control lanes contained a mixture of 2 ng each of two non-radioactive T7 RNA polymerase transcripts of riboprobe clones A and B (see Materials and Methods). Following electrophoresis the nucleic acids were transferred to a charged nylon membrane as described in Materials and Methods. Panel A shows an autoradiogram of a filter hybridized with the T3 RNA polymerase transcript of riboprobe A, containing mtDNA sequences between CSB 2 and the major 5′ ends of D-loop DNAs, as described in Materials and Methods. Panel B shows an autoradiogram of a filter hybridized with the T3 RNA polymerase transcript of riboprobe B, containing mtDNA sequences between CSB 2 and the mtDNA promoters, as described in Materials and Methods. The antisense RNA hybridization signals in lanes C are labeled on the right of panel B as 272 nucleotides (an antisense transcript of probe B) and 193 nucleotides (an antisense transcript of probe A). Some of the observed cross-hybridization of these probes may result from their common vector-derived sequences. Lane M in panel B contains mobility markers of end-labeled Msp I fragments of pBR322 ranging in size from 622 to 90 nucleotides.

oligonucleotide A hybridized to a single RNA approximately 300 nucleotides in size (Figs. 2A and 3A). The data presented below indicate that this 300 nucleotide RNA is not identical to the largest RNA detected with the 'upstream' probes (riboprobe B and oligonucleotide probe B).

Mapping the termini of major RNAs in the origin region

As noted above, the most abundant RNAs in the origin region map upstream of CSB 2 and hybridize to oligonucleotide B (Fig. 3B). We have previously reported the results of primer extension using oligonucleotide B as a primer (3). This experiment revealed three major 5′ ends of RNAs that hybridize to oligonucleotide B. These termini map to LSP 1, LSP 2B and an apparent processing site at map residue 864. We suggest that these three 5′ ends correspond to the three RNAs detected in the Northern blot hybridization in Fig. 3B. If this is correct, all three RNAs could share a common 3′ end near CSB 2. We performed an S1 mapping experiment to detect 3′ ends of RNAs in this region. The results, shown in Figure 4, indicate that the predicted 3′ end at CSB 2 is by far the most abundant 3′ end represented in mitochondrial RNA in this region. This 3′ end occurs at residue 682 ± 2, on the promoter-distal side of the prominent GC stretch in CSB 2. We note that a considerable number of additional RNA 3′ ends are detected in this experiment. Some of this heterogeneity may result from cleavage by S1 nuclease in AT rich sequences in this region or from the mapping of termini of RNA degradation intermediates. However, it is noteworthy that this experiment

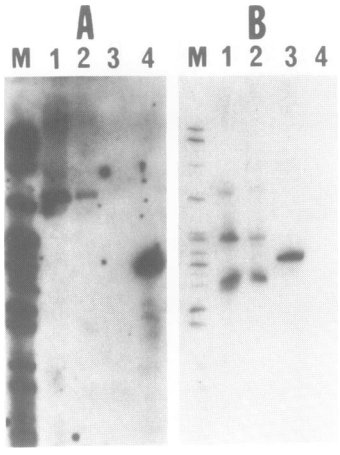

Fig. 3. Identification of origin region transcripts with oligonucleotide probes. Autoradiograms in panels A and B show the results of blot hybridizations using 5′ end-labeled oligonucleotides A and B, respectively, to probe identical filters containing mitochondrial nucleic acid samples. Samples were subjected to electrophoresis on a denaturing 6% PAGE-urea gel and blotted onto a charged nylon membrane as described in Fig. 2 and Materials and Methods. On each filter, the lanes labeled M contained end-labeled Msp I restriction fragments of pBR322 ranging in size from 622 to 90 nucleotides; lanes 1 and 2 contained 10 and 5 μg aliquots of total mitochondrial nucleic acid; lanes 3 and 4 contained control RNAs generated by *in vitro* transcription of linearized pBS plasmids containing short mtDNA inserts. The RNA in lanes 3 was a 178-nucleotide T7 RNA polymerase transcript of a pBS plasmid carrying mtDNA residues 805 to 968. The RNA in lanes 4 was a 193-nucleotide T7 RNA polymerase transcript of a pBS plasmid carrying mtDNA residues 541 to 673.

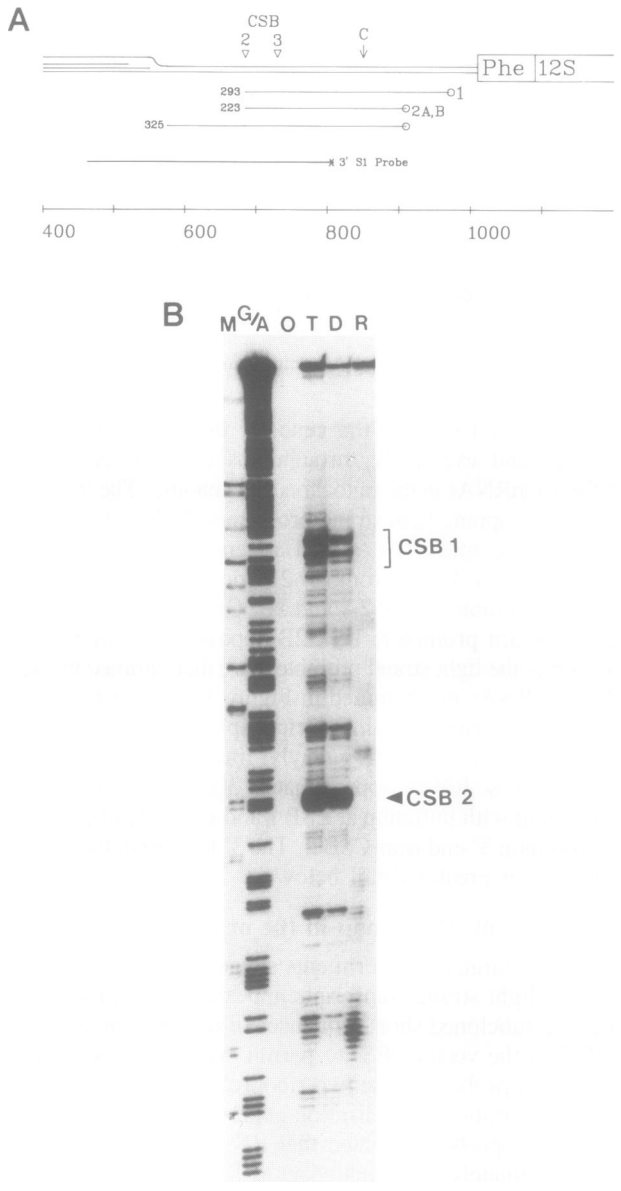

Fig. 4. S1 mapping of 3′ ends of RNAs in the origin region. A. Diagram of the position of the S1 probe. The probe used in this experiment contained mtDNA residues 460 to 804 and was labeled at the 3′ end at residue 804 as described in Materials and Methods. B. An autoradiogram of a denaturing polyacrylamide gel analysis of S1 digestion products is shown. The probe was either mock hybridized alone (lane 0), hybridized to total mitochondrial nucleic acids (lane T), to mitochondrial nucleic acids treated with DNase I (lane D) or to mitochondrial nucleic acids treated with RNase A (lane R). Hybridization mixtures were treated with nuclease S1 and the products analyzed on a 6% polyacrylamide gel containing urea. Markers in lane M include end-labeled Msp I fragments of pBR322 ranging in size from 309 to 67 nucleotides. Markers in lane G/A include fragments of the probe DNA generated by cleavage at purine residues.

did not detect any significant signal for potential RNA 3′ ends near CSB 3. This is a significant negative result, since we can account for all of the major RNA species detected by Northern hybridization without postulating RNA processing at CSB 3. This is discussed in greater detail below.

In order to map the 5′ termini of RNAs lying downstream of CSB 2, we performed an additional primer extension using oligonucleotide A, as shown in Figure 5. The major 5′ end identified in Figure 5 maps to LSP 2. This is somewhat surprising, since the experiments described above showed that CSB 2 is a major RNA processing site. Although we had expected to observe a major RNA 5′ end at CSB 2, only a very weak signal is seen at this location. It is possible that even this weak signal at CSB 2 is an artifact, since the appearance of the sequencing ladder markers in this region suggests that the AMV reverse transcriptase used in primer extension may have difficulty in reading through this sequence. We also do not see a significant incidence of RNA 5′ ends at residue 864 in the population of RNAs containing sequences downstream of CSB 2. These results suggest that there is ordered differential processing of RNAs in the origin region. RNAs processed at CSB 2 may be subject to additional processing at nucleotide 864. The major RNAs that contain sequences downstream of CSB 2 are not processed at either residue 682 (CSB 2) or 864.

The primer extension results shown in Figure 5 can be integrated with the results of Northern blot hybridization performed with the same oligonucleotide in Figure 3A. These experiments reveal a 320 nucleotide RNA with a 5′ end at map residue 905, ie., at LSP 2. We have also observed minor primer extension products extending to LSP 1 and, on longer exposures of experiments similar to that shown in Fig. 3B, a minor Northern blot signal consistent with this 5′ end. These results predict that there should be RNA 3′ ends near residue 585. These predicted species have been observed in S1 mapping experiments with 3′ labeled probes as shown in Figure 4, where a cluster of RNA 3′ ends is observed in this region and designated 'CSB 1'. We employ the designation 'CSB 1' advisedly, since this sequence homology is not as well-conserved in *X. laevis* mtDNA as CSB 2 or CSB 3. The data in Figure 5 do not permit a precise mapping of RNA 3′ ends in the CSB 1 region since these ends are located at a considerable distance from the labeled end of the S1 probe. This region is very close to the 5′ ends of stable D-loop DNAs

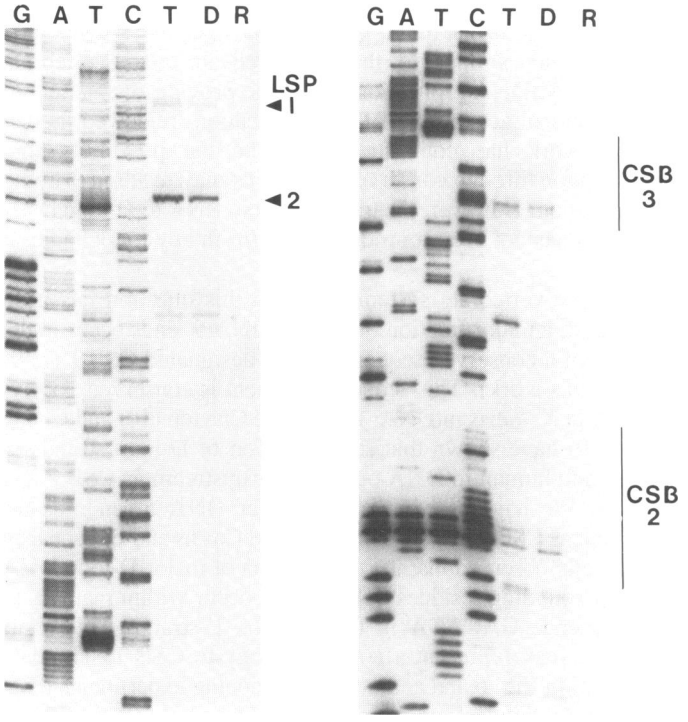

Fig. 5. Identification of 5′ ends of RNA's that hybridize to oligonucleotide A. An autoradiogram is shown of a denaturing 6% polyacrylamide gel analysis of products of primer extension reactions using the 5′ end-labeled oligonucleotide A shown in Fig. 1 as a primer. The right and left hand panels show the shorter and longer primer extension products, respectively, observed with different periods of electrophoresis. The mitochondrial nucleic acids used as templates for primer extension were either DNase I-treated (lanes D), RNase A-treated (lanes R) or untreated (total nucleic acid, lanes T). To generate homologous sequence markers, the same labeled oligonucleotide A primer was extended in the presence of dideoxynucleotides on a single-stranded M13 clone containing the mtDNA origin region to provide the sequence ladders in lanes labeled G, A, T and C.

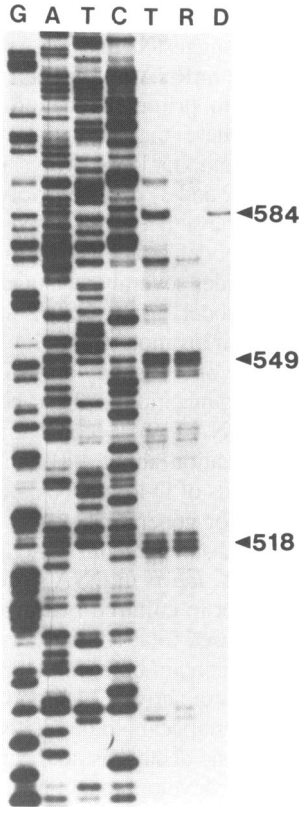

Fig. 6. Identification of 5′ ends of RNAs immediately preceding the 5′ ends of D-loop DNAs. An autoradiogram is shown of a denaturing 6% polyacrylamide gel analysis of products of primer extension reactions using the 5′ end-labeled oligonucleotide D shown in Fig. 1 as a primer. The primer extension reactions included either total mitochondrial nucleic acids (lane T), or mitochondrial nucleic acids treated with DNase I (lane D) or RNase A (lane R). To generate homologous sequence markers, the same labeled oligonucleotide D primer was extended in the presence of dideoxynucleotides on a single-stranded M13 clone containing the mtDNA origin region to provide the sequence ladders in lanes labeled G, A, T and C.

(6; see Fig. 1). We present additional data below to map DNA and RNA ends in this region.

Mapping RNA termini near the 5′ ends of D-loop DNAs

Cairns et al. (6) directly labeled 5′-OH ends of D-loop DNAs as a preliminary effort to map the D-loop in *X. laevis* mtDNA. This experiment identified the major D-loop DNA ends at residues 519±1 and 549±1 diagrammed in Figure 1 above. Since we detected evidence for RNA 3′ ends in this region, we employed primer extension with oligo D to map both RNA and DNA 5′ ends in the immediate vicinity of the 5′ ends of the major D-loop DNA species. The results, shown in Figure 6, present a complex array of RNA and DNA 5′ ends. These are indicated with reference to the sequence of the mtDNA H-strand in Figure 7. DNA ends are revealed by primer extension on mitochondrial nucleic acids treated with RNAse A. The primer extension results reinforce the observation by Cairns and Bogenhagen (6) that the major D-loop DNA ends occur in clusters surrounding residues 519 and 549, with a minor cluster surrounding residue 533. There are faint primer extension signals that may represent rare D-loop DNA ends further upstream, most noticeably at residue 571. However, we recall that the data shown in Figure 5 provided no evidence for DNA ends as far upstream as CSB 2 or CSB 3. Only a single significant primer extension product was observed when the template mitochondrial nucleic acid was treated with DNase. This is an RNA 5′ end at residue 584.

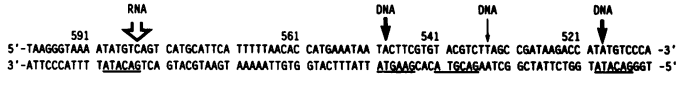

Fig. 7. Sequence of mtDNA from residues 511 to 600. The sequence is shown with the mtDNA H-strand as the upper strand. Arrows denote the DNA and RNA 5′ ends observed in Fig. 6 and discussed in the text. Sequences in the L-strand matching the GACPuTA at a minimum of 5 of 6 positions are underlined.

However, we note that the intensity of this signal is reduced in comparison to that in lane T, where total mitochondrial nucleic acid was used as the template. It is possible that this reduction in signal intensity may reflect the fact that some RNA 5′ ends are covalently linked to DNA. There are additional 5′ ends represented in lane T that were lost on treatment with either RNAse or DNAse. These could also represent nascent strands with RNA linked to DNA.

DISCUSSION

Summary of RNA mapping data

In this paper, we have presented a detailed analysis of the RNAs that map to the origin region of the *X. laevis* mtDNA genome.

Previous studies of RNA priming of replication of mouse and human mtDNA have suggested that RNAs synthesized by the transcriptionally active mtRNA polymerase (as distinct from an RNA primase) serve to prime replication of the H-strand of mtDNA (9, 10). In these mammalian systems, Clayton and coworkers have suggested that RNA processing at two conserved sequence blocks (CSB 2 and 3) may be involved in priming DNA replication (13). We have been interested in determining the extent to which this model is applicable to *X. laevis* mtDNA. *X. laevis* mtDNA provides several advantages as a model system in which to test this model. First, CSB 2 and 3 are interposed between the light strand promoters and the 5′ ends of stable D-loop DNAs in the same order as they occur in human and mouse mtDNA. This same organization is observed in rat mtDNA (5), but not in bovine mtDNA (19). Second, CSB 2 is found in *X. laevis* mtDNA at a location about 140 nucleotides away from the nearest major 5′ ends of D-loop DNA strands. This provides a substantial distance for generating probes that can be used to study the fate of RNAs that map in this region. However, it should be recognized that the *X. laevis* mtDNA system has considerable differences with respect to cultured mammalian cell lines. Our mapping studies have used total RNA isolated from the ovaries of mature frogs which contain both immature oocytes, in which mtDNA synthesis is quite active (7, 8) and mature oocytes, in which mtDNA synthesis appears to be relatively less active. The rate of RNA synthesis and decay has not been thoroughly studied as a function of oocyte development. Physiological differences between individual animals might complicate such experiments. We are not aware of any detailed studies of the half lives of mtRNAs in *X. laevis* oocytes. Our mapping studies have been confined to RNAs that have no coding function and no clearly defined structural role. Thus, the mapping data we have generated may include some predominant RNA 5′ and 3′ ends that are involved in RNA degradation. Despite this long list of caveats, the RNA mapping data we have presented permit the identification of a discrete set of RNAs in the origin region of the *X. laevis* mtDNA, as indicated in Table I. We consider that this data provides a significant background within which the long-distance RNA priming model can be evaluated in this sytem.

All of the RNAs we have identified appear to result from processing of primary transcripts that emanate from the previously characterized mtDNA promoters (2, 3, 4). Two of the major RNAs that hybridize to oligonucleotide B have 5′ ends at LSP 1 and 2, as does the single major RNA that hybridizes to oligonucleotide A. RNA ends occur at a few principle sites. A large fraction of RNAs in the origin region have 3′ ends at CSB 2, although CSB 3 does not appear to function as an RNA processing site for *X. laevis* mtRNA. RNAs upstream of CSB 2 are approximately 10- fold more abundant than downstream RNAs. Indeed, RNAs downstream of CSB 2 are sufficiently rare that we cannot rule out the possibility that this site serves as a leaky terminator of transcription. However, this site is not an efficient terminator of transcription from short linear fragments of mtDNA in reactions using highly purified mitochondrial RNA polymerase and a single initiation factor (Gokal and Bogenhagen, unpublished observation). It remains a possibility that termination occurs at this site in vivo and might be observed under other conditions.

How are D-loop DNA strands initiated? The exact mechanism for initiation of H-strand DNA replication in *X. laevis* remains unclear. As noted above, the general organization of sequence elements suggests that the *X. laevis* mtDNA genome may employ a mechanism similar to that observed in other vertebrate mtDNAs. MtDNA replication appears to provide an example of transcriptional activation of DNA replication (reviewed in 16). Recent work has concentrated on the the possibility that transcription through conserved sequences may result in priming of replication. However, the transition from RNA to D-loop DNA synthesis has not been reproduced *in vitro* in any mitochondrial system.

In every vertebrate system in which this question has been addressed, a major fraction of D-loop DNA 5′ ends occur in the vicinity of a conserved sequence block designated CSB 1 (5, 9, 10, 19; this work). This sequence element is conserved poorly, if at all, in *X. laevis* mtDNA. Chang and Clayton (10) and Chang et al., (9) have shown that some fraction of D-loop strands in mouse and human mtDNA occur as far upstream as CSB 2 and CSB 3. We have seen no evidence for DNA 5′ ends as far upstream as CSB 2 or CSB 3 in *X. laevis*. Cairns and Bogenhagen (6) initially observed that the two clusters of major D-loop DNA ends surrounding residues 518 and 549 occur within matches to the sequence GACATA in the mtDNA L-strand. This short sequence may represent a core homology to CSB 1. The data presented in Fig. 6 and additional S1 mapping experiments (data not shown) demonstrate a major RNA 5′ end at residue 584 of the mtDNA genome. It is interesting to note that this also occurs within a match to the GACATA motif (Fig. 7). It seems likely that processing at this site or at sites in the adjacent downstream sequence plays a role in generating primers for H-strand DNA replication. The experiment shown in Figure 6 revealed several examples of primer extension products observed in total mitochondrial nucleic acids but not after treatment with either DNase or RNase. This is exactly the behavior expected for nascent DNA strands bearing RNA primers, and is reminiscent of the evidence for RNA priming of DNA replication in mouse and human mtDNA (9, 10). We have examined the sequence in this region of the *X. laevis* mtDNA genome for obvious features that may be involved in this transition. The sequence between the processing site at 584 and the major D-loop DNA termini contains examples of short direct and inverted repeats that may play a role in the transition from RNA to DNA synthesis. However, additional experiments will be required to establish a clear model for RNA priming of DNA replication in *X. laevis* mtDNA.

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