Mapping of the Displacement Loop within the Nucleotide Sequence of Xenopus laevis Mitochondrial DNA*

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The mtDNA of the African frog, Xenopus laevis, has a triple-stranded displacement loop (D-loop) structure at the origin of heavy strand DNA replication. The major species of displacing strands has a length of 1670 nucleotides, approximately 3 times the length of mouse or human D-loop mtDNA strands. We report experiments that precisely map the termini of the D-loop strand within a revised sequence of the origin region. Analysis of D-loop mtDNA strands labeled in vitro at the 5' end using polynucleotide kinase and [γ-32P]ATP reveals microheterogeneity at the 5' end. The ends detected by this technique are located in the vicinity of several matches to a sequence element, denoted CSB-1, that is conserved in this location in several vertebrate mtDNA genomes. The 3' ends of D-loop mtDNA strands labeled in vitro by limited extension with avian myeloblastosis virus reverse transcriptase and [γ-32P]ATP are homogeneous. The sequence signals that may help specify the arrest of DNA replication at this site are discussed. The nucleotide sequence that we report for this region contains 53 discrepancies compared with a previously published sequence of this region of the Xenopus laevis mtDNA genome (Roe, B. A., Mia, D.-P., Wilson, R. K., and Wong, J. F.-H. (1985) J. Biol. Chem. 260, 9759-9774). Our sequence also contains a 142-nucleotide portion of the 5' end of the 12 S rRNA gene that was omitted from the sequence published by Roe et al.

Much of our current knowledge of the mechanism of replication of mitochondrial DNA (mtDNA) comes from studies of cultured animal cells (for review, see Clayton, 1982). Such cultured cells permit only a limited investigation of biological variability in the control of mitochondrial nucleic acid synthesis. The Xenopus oocyte presents an interesting system in which to study the developmental regulation and coordination between nuclear and mitochondrial nucleic acid metabolism. In unfertilized eggs of Xenopus, most of the DNA present is of mitochondrial origin (Dawid, 1965, 1966). The Xenopus oocyte requires several months to progress through a well ordered series of developmental stages (Dumont, 1972). mtDNA synthesis appears to occur at a maximal rate in immature oocytes that contain a large aggregation of mitochondria termed the Balbiani body (Al-Mukhtar and Webb, 1971; Billet and Adam, 1976). The high rate of mtDNA synthesis observed in these immature oocytes (Webb and Smith, 1977; Callen et al., 1980) occurs while nuclear DNA synthesis (other than amplification of rDNA (Brown and Dawid, 1968; Gall, 1968)) is inactive. The mtDNA content of the egg approaches 10^9 times the somatic cell complement before the rate of mtDNA replication decreases in late stages of oogenesis. This massive accumulation of maternal mtDNA permits the frog embryo to pass through several stages of early development without the synthesis of new mtDNA (Chase and Dawid, 1972).

A large proportion of the 17.6-kb Xenopus laevis mtDNA molecules (Cordonnier et al., 1982) contain the triple-stranded D-loop structure analogous to other vertebrate mtDNAs (Clayton, 1982). The D-loop structure represents a partial replication of the mtDNA H-strand. It has not yet been possible to determine whether the D-loop DNA serves to prime complete replication of the mtDNA H-strand, or may simply be an aborted product of an incomplete replication event. Studies of the replication of mammalian mtDNAs have suggested that the overall rate of mtDNA replication appears to be regulated by the frequency of H-strand DNA synthesis initiation and the efficiency of replication through sequences that sometimes terminate replication to yield the D-loop structure (Bogenhagen and Clayton, 1978; Clayton, 1982).

The occurrence and structure of the X. laevis mtDNA D-loop have been studied previously. Estimates of the D-loop frequency vary from 36% (Callen et al., 1983) to 78% (Hallberg, 1974) of the population of mtDNA molecules. The X. laevis mtDNA D-loop strand is considerably larger than the D-loop strands of mammalian mtDNAs. Electron micrographs show a single strand with a length of about 7% of the genome. The isolated single strand, with a sedimentation coefficient of 14 S, has a length estimated at 1550 nucleotides in various gel electrophoresis systems (Gillum and Clayton, 1978; Rastl and Dawid, 1979). The approximate position of the D-loop structure relative to the ribosomal RNA genes was established by examining electron micrographs of molecules cleaved by a restriction enzyme (Ramirez and Dawid, 1978) and refined by further restriction analysis (Rastl and Dawid, 1979; Cordonnier et al., 1982).

In this paper, we present a more detailed characterization of the 5' and 3' termini of the 14 S D-loop mtDNA as a necessary first step in understanding the control of mitochondrial nucleic acid metabolism in Xenopus. We have attempted to identify the termini of the D-loop mtDNA strands within the recently published sequence of the X. laevis mtDNA genome (Roe et al., 1986). However, we have found it necessary to revise the sequence of the heavy strand origin region in this report because we have found a significant incidence of discrepancies between our sequence and that previously reported.

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1 The abbreviations used are: kb, kilobase pair; AMV, avian myeloblastosis virus; NaDodSO4, sodium dodecyl sulfate, DTT, dithiothreitol.
by Roe et al. (1985). In the accompanying paper (Bogenhagen et al., 1986), we present data to identify the precise 5' termini of light and heavy strand transcripts within this sequence.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Determination of the Size of the D-loop DNA Displacing Strand—In order to map the ends of mature D-loop mtDNA strands within the nucleotide sequence (see Miniprint Supplement), we have analyzed samples of D-loop DNA strands labeled at the 5' and 3' termini. The sizes of the directly labeled single strands were determined by electrophoresis on a 1% agarose gel. The autoradiogram in Fig. 1 revealed a major species estimated as 1670 nucleotides in length along with minor species as short as 1550 nucleotides. The same size distributions are found for both 5' and 3' end-labeled samples and for 14 S strands detected by hybridization in Southern blotting experiments (data not shown).

Mapping the 5' Termini of Stable D-loop Strands—As noted above, we employed the end-labeling method used by Gillum and Clayton (1978) to identify the 5' ends of mouse and human D-loop mtDNAs (see "Experimental Procedures") to label the 5' ends of Xenopus mtDNA D-loop strands. The mtDNA used for these experiments was enriched for molecules containing D-loops by sedimentation (see "Experimental Procedures"). With mtDNA purified in this manner, most of the radioactive label incorporated represented label in the D-loop DNA strands as shown in Fig. 1. To locate the sites of 5' labeling with respect to the sequence, the DNA was recut with a number of restriction enzymes. We were concerned

with the possibility that the displacing strand might be lost from the D-loop containing molecules by branch migration during this restriction enzyme digestion. To increase the likelihood that the D-loop DNA strands would be duplexed for cutting with restriction endonucleases, we included single strands of a clone with L-strand sequences 1 to 400 in all reactions subsequent to kinase labeling.

Fig. 2 shows that multiple discrete cleavage products were observed with each of the enzymes *AluI*, *MspI*, *HaeIII*, and *Sau3A*. These enzymes have cleavage sites at residues 127, 283, 436, and 460, respectively, within the sequence shown in the Miniprint Supplement. In each case, cleavage of the D-loop DNA was apparently incomplete, since some high molecular weight DNA similar in size to the uncut end-labeled DNA was observed. The major end-labeled fragments observed in Fig. 2A upon recutting with *AluI* are approximately 400 and 430 nucleotides (lanes 3 and 5). The major species observed upon cleavage with *MspI* (lanes 2 and 4) are 235 and 265 nucleotides in length. Since the *AluI* and *MspI* sites are
separated by 156 nucleotides, the sizes of the major end-labeled fragments generated by these two enzymes are consistent with heterogeneity in the 5' ends of D-loop mtDNA. More accurate identification of the sites of end labeling is provided by recutting with enzymes closer to the 5' ends, as shown by the fragments cut by the enzymes HaeIII and Sau3A in Fig. 2B. HaeIII digestion produced fragments 112 and 80 nucleotides in length, labeled A and B in lane 3 of Fig. 2B. These fragments appear to result from cutting of the same D-loop strands that were cut by MspI to generate the fragments labeled A and B in lanes 2 and 4 of Panel A. Sau3A digestion produced a similar family of fragments, with each fragment shortened by about 24 nucleotides in comparison to the respective HaeIII fragment. We conclude that the two predominant stable 5' termini revealed by this labeling strategy occur at or near nucleotide residues 516 and 548 in the sequence shown in the Miniprint Supplement. The heterogeneity in location of these 5' termini probably contributes to the size heterogeneity for D-loop strands observed in Fig. 1. Additional lower molecular weight D-loop DNA strands seen in Fig. 1 may be represented by the shorter MspI and AluI fragments derived from end-labeled D-loop strands in Fig. 2A. These more rapidly migrating species have not been investigated in detail.

Mapping the 3' Termini of Stable D-loop DNA Displacing Strands—The strategy employed to determine the 3' termini of D-loop strands was similar to that used above to map 5' ends. AMV reverse transcriptase was used to extend the 3' ends. AMV reverse transcriptase was used to extend the 3' end-labeled D-loop strands in Fig. 3, lane 3) of Panel A. Sau3A digestion produced a similar family of fragments, with each fragment shortened by about 24 nucleotides in comparison to the respective HaeIII fragment. We conclude that the two predominant stable 5' termini revealed by this labeling strategy occur at or near nucleotide residues 516 and 548 in the sequence shown in the Miniprint Supplement. The heterogeneity in location of these 5' termini probably contributes to the size heterogeneity for D-loop strands observed in Fig. 1. Additional lower molecular weight D-loop DNA strands seen in Fig. 1 may be represented by the shorter MspI and AluI fragments derived from end-labeled D-loop strands in Fig. 2A. These more rapidly migrating species have not been investigated in detail.

Mapping the 3' Termini of Stable D-loop DNA Displacing Strands—The strategy employed to determine the 3' termini of D-loop strands was similar to that used above to map 5' ends. AMV reverse transcriptase was used to extend the 3' end of the displacing strand of D-loop DNA by one or a few nucleotides. The extent of elongation was controlled by limiting the supply of deoxynucleotide precursors. The 3' end-labeled D-loop DNA molecules were then cleaved with restriction enzymes and the resulting fragments were separated on denaturing gels (Fig. 3). The undigested end-labeled D-loop strands (Fig. 3, lane 3) were not accurately sized under these gel conditions. EcoRI cleavage produced a single fragment about 178 nucleotides long (Fig. 3, lanes 1 and 4) when the D-loop strands were labeled with either [α-32P]dATP plus [α-32P]dCTP or with [α-32P]dATP plus [α-32P]dCTP in the presence of nonradioactive dTTP. The use of nonradioactive dGTP in place of dTTP did not alter the pattern of results appreciably (data not shown). Cleavage with MboII resulted in a fragment of approximately 370 nucleotides (lane 5), consistent with the presence of an MboII cleavage site in genomic mtDNA (although not in pXlm 32) 196 nucleotides away from the EcoRI site. Thus, we conclude that the D-loop strand of X. laevis mtDNA has a unique 3' terminus located at a distance of slightly less than 178 nucleotides away from the EcoRI site. In this respect, the X. laevis mtDNA D-loop strand resembles the human mtDNA D-loop strand in having one major 3' termination site (Doda et al., 1981). The 3' terminus of D-loop strand occurs approximately 15 nucleotides away from tRNAPro.

**FIG. 3. Cleavage of 3' labeled D-loop strands reveals a unique 3' terminus.** An autoradiogram of a restriction enzyme analysis of 3' labeled D-loop mtDNA is shown. D-loop-containing mtDNA was 3' labeled as described under "Experimental Procedures" with deoxynucleotide mixtures containing [α-32P]dATP and [α-32P]dCTP either without (for lanes 1 and 3) or with (lanes 4 and 5) nonradioactive dTTP. The end-labeled mtDNA is shown without restriction enzyme digestion in lane 3. Lanes 1 and 4, DNA cleaved by EcoRI. Lane 5, DNA cleaved by MboII. Lane 2, MspI fragments of pBR322 ranging in size from 622 to 76 nucleotides (Sutcliffe, 1978).

**DISCUSSION**

The Nucleotide Sequence of the X. laevis mtDNA Origin Region—We present a revised sequence for the origin region of X. laevis mtDNA, derived from the plasmid pXlm 32 (see Miniprint Supplement). This sequence differs from that previously published for pXlm 31 by Wong et al. (1983) and Roe et al. (1985). An additional sequence of pXlm 31 has recently been published by Dunon-Bluteau et al. (1985). The discrepancies among these three sequences are considered in Table 2 (see Miniprint Supplement). Our sequence and that of Dunon-Bluteau et al. (1985) are in agreement in the 1497-nucleotide region surrounding the 5' portion of the D-loop. We have also determined the sequence of pXlm 31 in a 547-base pair MspI fragment spanning residues 638 to 1185 (Fig. 2 in Miniprint Supplement). This sequence was identical with the sequence we determined for pXlm 32. Our sequence and that of Dunon-Bluteau et al. (1985) share 45 discrepancies with the sequence reported by Roe et al. (1985). We consider that the small number of discrepancies between our sequence and that of Dunon-Bluteau et al. (1985) in the 3' portion of the D-loop may result from actual variations between pXlm 31 and pXlm 32. As noted in Table 2 (Miniprint Supplement), two of the nine differences have been shown to create restriction site polymorphisms that have been documented by restriction analysis of pXlm 31 and pXlm 32 (data not shown). It is interesting to note that these differences are located in the distal end of the D-loop region, described by Upholt and Dawid (1977) as a region of high evolutionary instability. In contrast, we have observed numerous sites for cleavage by HhaI, EcoRI, DdeI and Sau3A that are apparently incorrectly represented in the sequence of Roe et al. (1985). Thus, we consider that the sequence shown in the miniprint supplement is correct for both pXlm 31 and pXlm 32 in the region showing agreement with the sequence of Dunon-Bluteau et al. (1985).

Due to the frequency of insertions and deletions in our sequence in comparison with that of Roe et al. (1985), we have employed a different numbering system. Since we are particularly interested in the sequences between the 5' end of D-loop strands and the tRNAPro gene, a region where the sequences of pXlm 31 (Dunon-Bluteau et al., 1985) and pXlm 32 are in agreement, we have begun numbering the sequence at a Sau3A site within the D-loop. This is analogous to the numbering system employed by Anderson et al. (1981) for the sequence of the human mtDNA genome.

Structure of the 5' End of X. laevis 12 S rRNA—We have determined that the sequence of endogenous mitochondrial 12 S rRNA contains the additional 142 nucleotides found in the sequence of the cloned mtDNA (Fig. 3 in Miniprint
We have also sized 12S rRNA from both human and X. laevis mitochondria following electrophoresis of total RNA and Northern hybridization with human and X. laevis mtDNA probes homologous to the 5' end of the 12S rRNA gene (data not shown). The results confirm that the two RNAs are almost identical in size, as would be predicted by the addition of the 142 bases to the 519 of the Roe et al. (1985) sequence. We also observed extensive cross-hybridization between the two probes and the two RNAs. A computer alignment between 174 nucleotides of the 5' regions of the X. laevis and human 12S rRNAs shows 116 matches, or 67% homology.

**Determination of the Size of the D-loop Displacing Strand and Location of the Major 5' and 3' Termi**n within the Nucleotide Sequence—Early studies recognized that the D-loop strand of X. laevis mtDNA was much larger than that of other vertebrates (Hallberg, 1974; Ramirez and Dawid, 1978; Rastl and Dawid, 1979). Our preliminary Southern hybridization of labeled L-strands identified the size of the major D-loop species to be about 1650 nucleotides (data not shown). This major species has also been identified by 5' and 3' labeling of its termini (Fig. 1). This size is somewhat longer than previous estimates (Gillum and Clayton, 1978; Rastl and Dawid, 1979). However, these earlier studies either used incomplete denaturing gel conditions or did not employ sequenced gel markers. Our estimate of the size of the directly labeled X. laevis D-loop strands is in excellent agreement with the locations of the 5' and 3' termini that we have mapped for the labeled strands. When the 5' and 3' termini are mapped on a sequence including our revisions, the most abundant D-loop species extend from approximately nucleotide 548 to approximately -1128, giving a size of about 1670 nucleotides.

The short D-loop H strands have been well studied in mouse and human cells. Gillum and Clayton (1978) identified three major species of D-loop DNA in human cells and four major species in mouse cells. Varying degrees of microheterogeneity have been encountered at both 5' and 3' ends of the D-loop DNA strands (Brown et al., 1978; Tapper and Clayton, 1981; Doda et al., 1981). Our data indicate that the heterogeneity in size of the X. laevis D-loop DNA results from variable 5' termini. The major class of D-loop DNA strands observed in Fig. 1, with lengths of approximately 1640 to 1670 bases, have 5' ends near residues 516 and 548 (see the sequence in the Miniprint Supplement). The minor population of D-loop DNA strands observed in Fig. 1, which are as short as 1550 bases, probably have 5' ends seen as minor Mspl cleavage products 100 to 160 nucleotides in length in Fig. 2A.

We have identified the 5' ends of the D-loop strands from mature oocyte mtDNA by kinase labeling of the 5' nuclease without the use of phosphatase to remove any potential 5'-terminal phosphate end groups. This procedure would be expected to efficiently label only 5'-OH termini. This suggests that these are processed ends rather than exact initiation sites. Gillum and Clayton (1979) showed that a large fraction of mouse mtDNA D-loop strands contain short oligoribonucleotide regions at their 5' ends. This observation is consistent with an RNA priming model, but is not sufficient to identify the true initiation sites or to determine whether D-loop DNA synthesis is primed by short oligoribonucleotides, as might be expected for a primase activity. More recent experiments reported by Chang and Clayton (1985) have raised the possibility that human D-loop mtDNA synthesis is primed by RNAs initiated at an L-strand transcriptional promoter located over 100 nucleotides upstream from the stable D-loop 5' ends. It might be expected that D-loop DNA's bearing complete, unprocessed 5' termini would be rather rare. It has not yet been possible to define the 5' terminal structure of nascent Xenopus D-loop DNA strands in sufficient detail to confirm the model proposed for human mtDNA replication by Chang and Clayton (1985). The 5' mapping information presented in this paper is intended to serve as a reference for more detailed mapping experiments that may become feasible in the future.

**Nucleotide Sequences Involved in the Control of D-loop DNA Synthesis**—The 5' termini of the two major 14 S strands occur near residues 516 and 548 of the sequence in the Miniprint Supplement. A number of other minor termini are also observed. The most significant of these appears to have a 5' end at about residue 536. We have analyzed the sequences in this region for relationships between these major 5'-terminal sequences and for any potential relationship with the sequences near the 5' termini of other mtDNA D-loop strands. Walberg and Clayton (1981) have identified a conserved sequence block, designated CSB-1, shared by the mouse, human, and rat mtDNA genomes. This block occurs in the vicinity of the 5' ends of D-loop mtDNA strands in each system, but is not as extensive nor as well conserved as other homologous sequences noted by Walberg and Clayton (1981), CSB-2 and CSB-3. Sequences homologous to CSB-1 occur near the 5' termini of stable Xenopus D-loop DNA strands as well. The sequences GACATA may serve as a potential Xenopus version of CSB-1. Wong et al. (1983) identified one match to CSB-1 at a location corresponding to residues 585 to 590 in our numbering system. However, we note that several matches to this short consensus occur near the 5' ends of D-loop strands. In particular, matches to CSB-1 occur surrounding the two major 5' termini: nucleotides 514 through 520 have a 7/7 match and nucleotides 545 though 551 have a 5/7 match. An additional 6/7 match at residues 536 to 541 may correspond to a minor 5' end evident in Fig. 2. A similar arrangement exists in mouse and human mtDNA. The major 5' end of the human D-loop strands is located within the sequence GAA-CATA and is 38 bases downstream from the “canonical” CSB-1 sequence noted by Walberg and Clayton (1981). The major 5' termini of mouse D-loop strands are adjacent to CSB-1 (Gillum and Clayton, 1979; Chang et al., 1985). We consider that the CSB-1 homology may be part of a recognition sequence that specifies stable 5' termini for D-loop DNA's. However, for the reasons noted above, this is not likely to be a primary DNA initiation site or a site for priming replication.

The experiment reported in Fig. 3 shows that the 3' terminus of D-loop strands occurs uniquely at a site approximately 175 nucleotides away from an EcoRI cleavage site. Ramirez and Dawid (1978) first mapped an EcoRI site near the 3' end of the D-loop DNA by electron microscopy of mtDNA molecules spread in formamide. When our more precise mapping information is used to locate the termination site within the revised nucleotide sequence shown in Fig. 2 of the Miniprint Supplement, it is apparent that termination occurs in the region preceding the tRNAp gene, as in other vertebrate systems (Clayton, 1982). Our analysis of the sequence in this region reveals an additional 30 bases in the D-loop region near the tRNAp gene which is not found in the sequence reported by Roe et al. (1985). Without this revision of the sequence, the location of the 3' end of the D-loop DNA would occur at an anomalous site, within tRNAp. The 30-base-pair insertion occurs at one edge of a repeated sequence identified by Roe et al. (1985) and extends the direct repeat to a match of 65 out of 67 nucleotides.

Doda et al. (1981) analyzed the sequences near the termini of human and mouse D-loop strands to investigate the possi-
bility that DNA synthesis termination may be signaled by a particular DNA sequence. No significant homology was found in the immediate vicinity of 3' ends of D-loop DNA strands. However, they identified a 12-15-nucleotide sequence approximately 50 nucleotides upstream from each site of termination of D-loop strands in mouse and human mtDNA. They argued that the correlation between the number of these sequences and the number of stop points suggests a functional role for these sequences in arresting D-loop synthesis. For X. laevis D-loop strands, we also find no significant homology of the terminal sequences to mouse or human mtDNA. However, we find matches of limited homology to the consensus sequence of Doda et al. (1981) near the 3' end of X. laevis mtDNA, as shown in Table 1. This homologous sequence may play a role in termination of D-loop DNA synthesis. However, the fact that this sequence is found in two copies does not correlate with the identification of one 3' terminus for X. laevis D-loop DNA. In addition, the location of these sequences with respect to the termination site is different from that predicted by the model of Doda et al. (1981). The single termination site is located 62 and 157 nucleotides away from these potential termination signals. Additional experiments will be required to define the sequences responsible for termination of D-loop DNA synthesis. The mapping experiments reported here should help to establish the Xenopus oocyte as a suitable experimental system for further investigation.

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Continued on next page.
**Supplement to: Mapping of the Displacement Loop within the Nucleotide Sequence of Xenopus laevis Mitochondrial DNA**

**Isolation of Mitochondrial DNA**: Mitochondria were isolated from ovaries of mature Xenopus laevis (Ambystoma tigrinum) as described by Diederich (1971). The mitochondria were then resuspended in TE (10 mM Tris, pH 7.5, 1 mM EDTA) buffer containing 250 mM sucrose and 50 mM Tris, pH 7.5, and spun in a Beckman 55 Ti rotor at 35,000 rpm for 2 hr. The mitochondrial DNA was then isolated from the supernatant by extraction with phenol and purified by sucrose density gradient centrifugation.

**Enrichment of D-loop-containing mtDNA**: The mtDNA was digested with the restriction enzyme MspI (0.1U/µg DNA) and the fractions were separated on a 0.7% agarose gel. The D-loop was then isolated by gel extraction and purified by sucrose density gradient centrifugation.

**Location of intact D-loop Strands**: DNA from the pooled sucrose gradient fractions was purified and concentrated to 10 µg/µl by ethanol precipitation. The DNA was labeled directly with [3,13-32P]dATP (Amersham Corp.) and polyadenylic acid (Sigma) and [3,13-32P]dATP (Pharmacia) and polyadenylic acid (Sigma) and [3,13-32P]dATP (Pharmacia) and polyadenylic acid (Sigma) and [3,13-32P]dATP (Pharmacia) and polyadenylic acid (Sigma) and [3,13-32P]dATP (Pharmacia) and polyadenylic acid (Sigma) and [3,13-32P]dATP (Pharmacia) and polyadenylic acid (Sigma). The labeled DNA was separated by gel electrophoresis and visualized by autoradiography.

**Subcloning Strategies**: Subclones of X. laevis mtDNA were generated by cleavage with restriction enzymes and cloning into M13mp vectors. The labeled fragments were cloned into M13mp vectors and sequenced by the dideoxy method.

**Sequence of the heavy strand origin region of X. laevis mtDNA**: In order to map the extent of the D-loop region, a variety of fragments of the X. laevis mtDNA were excised from the plasmid pXIm 32 digested with 30 U of the restriction enzymes DdeI, MspI, and HinfI. The labeled fragments were then cleaved with restriction enzymes and subcloned into M13mp vectors. The labeled fragments were then sequenced by the dideoxy method.

**Fig. 1**: The genetic organization of the D-loop region of X. laevis mtDNA showing the extent of subcloning and sequencing. The triple-stranded D-loop tracts are positioned relative to the flanking regions of the X. laevis mtDNA. The RNA genes are transcribed from the H strand in the direction opposite to that of the D-loop tract. Several restriction enzyme sites are marked by a filled circle. A region of the D-loop tract containing the 32P-labeled disulfide bond was sequenced from pXIm 32. Arrows above the line denote the extent of sequences obtained from the D-loop tract. Nucleotide numbers correspond to the sequences in Fig. 2.

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**Results**: The sequence of the heavy strand origin region of X. laevis mtDNA was determined in order to map the extent of the D-loop DNA. A variety of fragments of the X. laevis mtDNA were excised from the plasmid pXIm 32 digested with 30 U of the restriction enzymes DdeI, MspI, and HinfI. The labeled fragments were then cleaved with restriction enzymes and subcloned into M13mp vectors. The labeled fragments were then sequenced by the dideoxy method.

**Fig. 2**: Sequence of the D-loop region. This sequence was obtained by sequencing the cloned fragments of pXIm 32. The sequence is drawn to scale in the direction opposite to that of the D-loop. Several restriction enzyme sites are marked by a filled circle. The sequence was determined by the dideoxy method using bacteriophage M13mp 10 vectors. A sad sulfate disulfide bond was sequenced from pXIm 32. Arrows above the line denote the extent of sequences obtained from the D-loop tract. Nucleotide numbers correspond to the sequences in Fig. 1.
Fig. 2 shows the sequence of 2458 nucleotides around the origin of mDNA replication for the plant mitochondria. The sequence shown is of the mtDNA strand, numbered with reference to a specific site within the D-loop region (coordinate 0 in Fig. 1). As shown in Table 2, we observe 35 positions at which our sequence does not agree with that of War et al. (1985) published by the anonymous authors. The differences are mainly nucleotide substitutions, insertions, and deletions. We also observed a deletion of 5 base pairs and two large insertions of 18 nucleotides each (Fig. 2 and Table 2). These insertions are discussed further below. Two differences noted at bases 457 and 465 locate a cluster of two mismatch sites identified by R. Marworth and H. Sibley (1985) and by Corcoran et al. (1985).}

**Table 2.** Identification of nucleotide differences between the sequence shown in Fig. 2 and that of War et al. (1985). Asterisks indicate residues where the sequences of War et al. (1985) agree with that of War et al. (1985) and differ from our sequence.

While the work was proceeding, similar findings led other investigators to sequence the same region from xaln 31 (War et al. 1985). The sequence in this region differs from that of War et al. (1985) and that of War et al. (1985) and whereas the other groups have sequenced xaln 31, we have focused on xaln 31 and xaln 32 data not shown. We have noted the discrepancies between our sequence and that of War et al. (1985) in Fig. 2 and Table 2.

**Size of the X, lambs 125 RNA.** The most significant discrepancy between our sequence and that of War et al. (1985) is a 162 base pair deletion in the sequence shown. The size of the deletion was confirmed by the authors of the clone used in our study. We have sequenced the entire X, lambs 125 RNA sequence except for the 162 base pair deletion. We have performed two rounds of sequencing to demonstrate that the 162 base the authors report in Fig. 2 are contained within the RNA. In addition, we have sequenced the entire X, lambs 125 RNA sequence except for the 162 base pair deletion. We have performed two rounds of sequencing to demonstrate that the 162 base pair deletion is contained within the RNA sequence. Our results are consistent with the authors of the clone used in our study.