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Template Sequences Required for Transcription of *Xenopus laevis* Mitochondrial DNA from Two Bidirectional Promoters

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Previous work from our laboratory has shown that transcription of *Xenopus laevis* mitochondrial DNA initiates both in vivo and in vitro from bidirectional promoters located between the gene for $tRNA^{Phe}$ and the 5′ termini of displacement loop DNA strands. A consensus sequence matching the octanucleotide ACGTTATA surrounds each transcription start site. In the present study, we used in vitro mutagenesis to define sequences required for specific transcription in vitro. First, cloned mitochondrial DNA templates generated by deletion mutagenesis were transcribed in vitro to define the limits of functional promoters. The bidirectional promoter located approximately 33 nucleotides upstream from the gene for $tRNA^{Phe}$, termed promoter 1, was studied in greatest detail. The results confirmed the hypothesis that the consensus octanucleotide sequence surrounding each start site is an essential promoter element. A duplex 18-base-pair oligonucleotide encoding the symmetrical promoter 1 region was synthesized and cloned in a plasmid vector. This synthetic oligonucleotide was sufficient to support bidirectional transcription. Point mutations within this oligonucleotide were used to identify critical residues within the consensus sequence.

Vertebrate mitochondrial DNA (mtDNA) genomes have been found to share a common pattern of gene organization. Each contains essentially the same set of genes coding for subunits of mitochondrial respiratory complexes and for a set of structural RNAs, rRNAs, and tRNAs sufficient to permit translation of the mitochondrial mRNAs on mitochondrial ribosomes. The ordering of these genes is precisely conserved for the vertebrate mtDNA genomes that have been sequenced, including the human (1), mouse (3), bovine (2), and *Xenopus laevis* (29) mtDNAs. Each of these vertebrate mtDNA genomes has one relatively large noncoding region that contains an origin of replication and start sites for transcription.

Two general approaches have been used to map promoters for transcription by the mtRNA polymerase. First, the 5′ ends of nascent RNAs have been mapped within the noncoding region separating the genes for rRNAs and $tRNA^{Phe}$ from the 5′ ends of the D-loop DNA strands in the human and *X. laevis* mtDNAs (10, 39). Second, functional promoter sequences have been defined by using cloned mtDNAs as templates for in vitro transcription by the homologous mtRNA polymerase. Several groups have studied the in vitro transcription of human mtDNA (6, 12, 17, 22–24, 35, 38). The promoter sequences have been designated HSP and LSP for promoters driving transcription of the heavy and light strands of mtDNA, respectively. Transcription of human mtDNA has been found to initiate within either of two conserved sequences separated by about 150 base pairs (bp). Murine mtDNA has a similar promoter organization (14, 15), although in this case there is a lack of clear homology between heavy- and light-strand promoters. In both mammalian systems, sequences located upstream from the sites of initiation have been found to have a major role in directing the activity of in vitro transcription.

The *Xenopus* mitochondrial system has been of particular interest since the discovery by Dawid (19, 20) that the large majority of total cellular DNA in *Xenopus* oocytes is of mitochondrial origin. We have begun experiments to study the developmental control of mitochondrial nucleic acid synthesis in oocytes. Transcription initiates within two regions of the mtDNA genome separated by about 60 bp (10). Each initiation site is surrounded by a sequence that is at least a seven of eight match to a consensus octanucleotide sequence, ACGTTATA. We have suggested that this consensus sequence is likely to be an element of a mitochondrial promoter and that the bidirectional character of *Xenopus* mitochondrial promoters results from the symmetrical arrangement of these consensus sequences (9). In the present study, we confirmed this hypothesis by using deletion mutagenesis to define the minimal sequences required for promoter activity in vitro. We also showed that a cloned 18-mer representing the promoter 1 region adjacent to $tRNA^{Phe}$ is sufficient to encode a bidirectional promoter and subjected this synthetic promoter to point mutagenesis.

MATERIALS AND METHODS

Materials. Exonuclease III, nuclease S1, and T4 DNA ligase used for deletion mutagenesis were obtained from New England BioLabs, Inc. (Beverly, Mass.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), respectively. Restriction endonucleases and the large fragment of DNA polymerase I were obtained from New England BioLabs. Sequencing chemicals were from Pharmacia, Inc. (Piscataway, N.J.). Radioisotopes were from ICN Pharmaceuticals, Inc. (Irvine, Calif.). Kodak XAR-5 X-ray film was used for autoradiography. The pBS- cloning vector was obtained from Stratagene (San Diego, Calif.).

Cloned mtDNA templates. Deletion mutants were derived from a cloned *Dde*I fragment containing residues 806 to 1050 of *X. laevis* mtDNA (11), designated DdeA2, which has previously been shown to contain two bidirectional promoters (9). All deletion derivatives were cloned in plasmid pUC (37). Deletion mutagenesis was performed on either *Eco*RI- or *Hin*dIII-digested DNA by sequential treatment with exonuclease III and nuclease S1 as described previously (8). Flush-ended DNAs deleted from the *Eco*RI site were recut with *Hin*dIII, gel isolated, and religated to M13mp9 cut with

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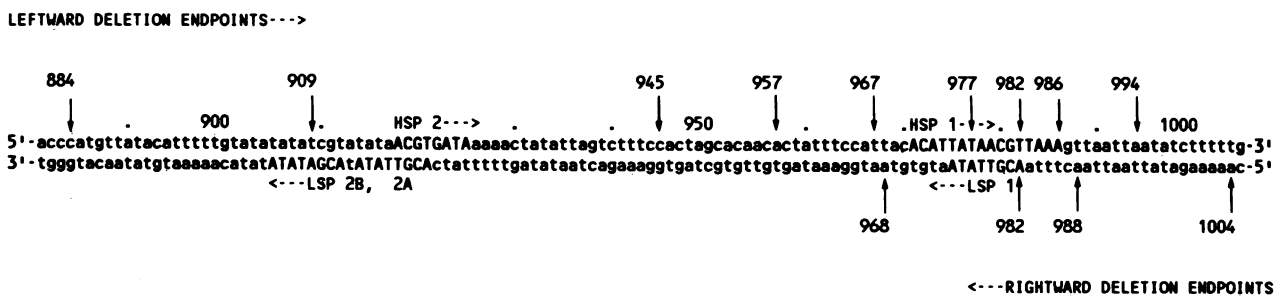


FIG. 1. *X. laevis* mtDNA promoter region. The sequence is shown for nucleotides 881 to 1005 of *X. laevis* mtDNA immediately preceding the gene for $tRNA^{Phe}$. The upper strand has the sense of the mtDNA light strand. Regions with at least seven of eight matches to the sequence ACGTTATA surrounding sites of in vitro transcription initiation are shown in upper case. The vertical arrows above the sequence indicate endpoints for deletions entering this sequence from the left. The vertical arrows below the sequence indicate endpoints for deletions entering this sequence from the right.

*Hin*dIII and *Hin*cII. DNAs deleted from the *Hin*dIII site were recut with *Eco*RI, gel purified, and religated to M13mp9 recut with *Hin*cII and *Eco*RI. Deletion endpoints were defined by chain terminator sequencing (31). The *Eco*RI-*Hin*dIII inserts of selected mutants were transferred to pUC9. Restriction fragments of these clones were used as templates for in vitro runoff transcription. Fragments were purified by electrophoresis on agarose gels, identified by UV shadowing or staining with ethidium bromide, and recovered by electroelution. DNA samples to be used as transcription templates were extracted first with phenol and then ether and were concentrated by ethanol precipitation. The yield of DNA fragments was estimated by electrophoresis on agarose minigels and by a quantitative fluorometric procedure (25).

Construction and mutagenesis of synthetic bidirectional promoter. An 18-bp region of mtDNA (see Fig. 5) was cloned in plasmid pBS− with additional flanking nucleotides required to adapt the termini to *Eco*RI and *Pst*I sites. Oligonucleotides were synthesized by phosphoramidite chemistry with a DNA synthesizer (either Systec Microsyn-1450A or Vega Coder 300 model) and were repurified by reverse-phase high-pressure liquid chromatography. To generate point mutations within the mtDNA region, we synthesized 20-mer oligonucleotides with mixtures of precursors inserted at several sites within the sequence. These degenerate oligonucleotides were hybridized to the 28-mer necessary to permit cloning in *Eco*RI-*Pst*I-cut pBS−. Five mixed oligonucleotides were generated to produce the mutations described here. Clones were screened by chain terminator sequencing (31) with the single-stranded form of the pBS- plasmid.

In vitro transcription. Transcription was performed with either of two transcription systems. First, deletion mutants were transcribed with a mitochondrial extract purified by chromatography on heparin-Sepharose and phosphocellulose as described by Bogenhagen and Yoza (9). This preparation contains both the mtRNA polymerase and a specific transcription factor. Second, both deletion mutations and point mutations were transcribed with a reconstituted system described in the accompanying paper (7). The two transcription systems provided similar results for the relative transcription efficiencies observed for deletion mutants. Transcription of templates with deletion mutations was performed with gel-purified restriction fragments. Transcription of templates with point mutations in the synthetic promoter was performed with total *Pvu*II digests of plasmid DNA. Transcripts were resolved by electrophoresis through polyacrylamide gels containing 8 M urea and detected by autoradiography as described previously (9). When indicated, relative transcription efficiencies were estimated by densitometry of autoradiograms with an LKB laser densitometer.

RESULTS

Deletion analysis of mitochondrial promoters. A recloned *Dde*I fragment of *X. laevis* mtDNA spanning residues 806 to 1050 of the sequence reported by Cairns and Bogenhagen (11) was used as a beginning point for deletion mutagenesis. The ability of this fragment to serve as a template for in vitro transcription has been described previously (9). This *Dde*I fragment contains two bidirectional promoter regions. One region, termed promoter 1, is located about 33 nucleotides upstream of the $tRNA^{Phe}$ gene, while a second bidirectional promoter, termed promoter 2, is located 60 nucleotides further upstream. The consensus sequences matching ACGTTATA surrounding initiation sites are shown in capital letters in Fig. 1. Altogether, there are five functional promoters within this fragment.

The first set of deletion mutations constructed approaches the mitochondrial promoters from the left, with the deletion endpoints indicated along the sequence in Fig. 1. It is important to emphasize that such a set of deletions can be considered 3′ deletions with respect to the light-strand promoters (LSP) and 5′ deletions with respect to the heavy-strand promoters (HSP).

The ability of deleted mtDNAs to serve as templates for transcription by the *X. laevis* mtRNA polymerase was tested by a runoff transcription assay performed as described by Bogenhagen and Yoza (9). In each case, the plasmids containing the cloned mtDNA fragments were digested with pairs of restriction enzymes selected so that the runoff transcripts produced from all five mitochondrial promoters would be distinguishable from one another by size. The runoff transcripts of the templates bearing leftward deletions are shown in Fig. 2. With this deletion approach, the heavy-strand transcripts from any transcriptionally active template remain constant in size, while the light-strand transcripts are progressively shortened as the extent of the deletion is increased. The results shown in Fig. 2 clearly indicate that the two regions contain separate bidirectional promoters. Both HSP 2 and LSP 2 were inactivated by deletion to residue 945, while the promoters in region 1 were active even after deletion to residue 967. Further deletion to residue 977 or beyond inactivated both HSP 1 and LSP 1.

Additional deletion mutations were generated to define further the extent of mitochondrial promoters in promoter

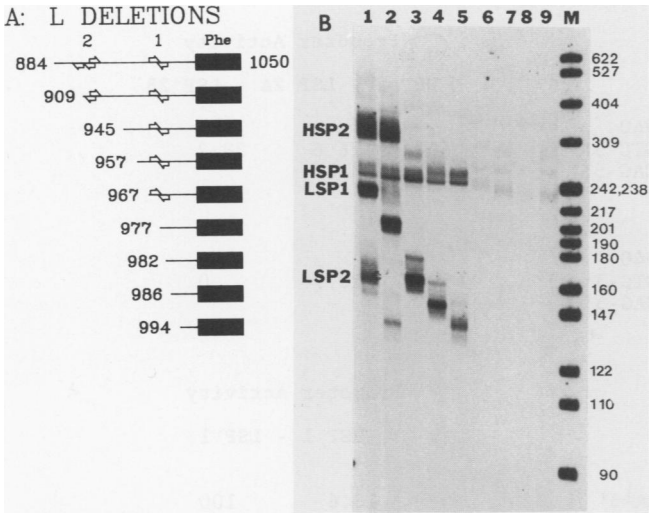


FIG. 2. Effects of leftward deletions on mitochondrial promoter activity. (A) Diagrams of deleted templates indicating the positions of the deletion endpoints with respect to the conserved sequence blocks surrounding transcription start sites. All the deletion mutants are flanked by identical *Hin*dIII and *Eco*RI site sequences on the left and right, respectively, and are cloned in pUC9 (37). The open arrows indicate matches of at least seven of eight residues to the conserved sequence ACGTTATA. (B) Autoradiogram of a polyacrylamide-urea gel electrophoretic analysis of in vitro transcripts of *Ban*I-*Bgl*I fragments of the pUC9 clones containing the *X. laevis* mtDNA inserts diagrammed in panel A. Lanes 1 to 9 contain the transcripts of templates ΔL884, ΔL909, ΔL945, ΔL957, ΔL967, ΔL977, ΔL982, ΔL986, and ΔL994, respectively. The RNAs synthesized from template ΔL884 are labeled on the left. With this deletion approach, RNAs synthesized from heavy-strand promoters have a constant size of 295 and 242 nucleotides for HSP 2 and HSP 1 transcripts, respectively. RNAs synthesized from light-strand promoters have sizes that decrease as the extent of deletion increases. Lane M contains markers of end-labeled *Msp*I fragments of pBR322 (36) with sizes in nucleotides indicated on the right.

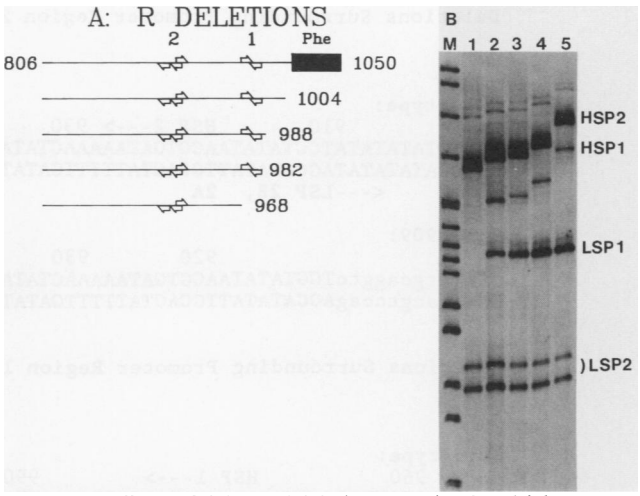


FIG. 3. Effects of rightward deletions on mitochondrial promoter activity. (A) Diagrams of deleted templates indicating the positions of the deletion endpoints with respect to the conserved sequence blocks surrounding transcription start sites. All the deletion mutants are flanked by identical *Hin*dIII and *Eco*RI site sequences on the left and right, respectively, and are cloned in pUC9 (37). The open arrows indicate matches to the conserved sequence ACGTTATA. (B) Autoradiogram of a polyacrylamide urea gel electrophoretic analysis of in vitro transcripts of *Nde*I-*Hin*dIII fragments of the pUC9 clones containing the *X. laevis* mtDNA inserts diagrammed in panel A. Lanes 1 to 5 contain the transcripts of templates ΔR968, ΔR982, ΔR988, ΔR1004, and ΔR1050, respectively. The RNAs synthesized from template ΔR1050 are labeled on the right. With this deletion approach, RNAs synthesized from light-strand promoters have a constant size of 185 nucleotides for LSP 1 and 128 and 118 for LSP 2A and 2B, respectively. RNAs synthesized from heavy-strand promoters have sizes that decrease as the extent of deletion increases. Lane M displays gel mobility markers of end-labeled *Msp*I fragments of pBR322 ranging in size from 622 to 90 nucleotides (Fig. 2).

region 1. This second set of deletions approaches the mitochondrial promoters from the right with deletion endpoints as indicated along the sequence in Fig. 1. The results obtained with these rightward deletion mutations are shown in Fig. 3. Deletion to residue 982, exactly to the limit of the LSP 1 consensus sequence, had only minor effects on transcription from HSP 1 or LSP 1. Further deletion to residue 968, removing both consensus sequences, eliminated all transcription from promoter region 1. Thus, we conclude that the consensus sequence ACGTTATA is the major element of an *X. laevis* mitochondrial promoter.

The results of deletion analysis were examined in light of the specific sequences required for promoter activity (Fig. 4). The most extreme leftward deletion that left some residual activity of promoters in region 2 was the deletion to residue 909. This deletion actually had little, if any, effect on the activity of HSP 2, but had a major effect on LSP 2. This result correlates well with the positions of the putative consensus promoter sequences within region 2. LSP 2 contained a direct repeat of the consensus sequence between residues 905 and 921 and supported the synthesis of two discrete RNAs that appear to initiate within these two repeats (Fig. 4). Deletion to residue 909 deletes the downstream half of LSP 2, designated LSP 2B by Bogenhagen and Yoza (9), leaving only the adjacent LSP 2A element intact and transcriptionally active (although only weakly so; see lane 2 of Fig. 2B).

Further evidence for the importance of the consensus sequence is offered by the analysis of more extreme leftward deletions affecting promoter region 1. The activities of both LSP 1 and HSP 1 were lost upon deletion of the 10 bp between residues 967 and 977. Deletion to residue 977 encroached on the consensus sequences for both promoters (Fig. 4). It is interesting that the juxtaposition of polylinker sequences adjacent to the mtDNA in L977 leaves the sequence ACGTTAGA at LSP 1. This seven of eight match to the consensus promoter sequence is not functional.

Sequences outside the core consensus can affect promoter activity. The in vitro transcription analysis of *X. laevis* mtDNA deletion mutants suggested that the core consensus sequence plays a dominant role in determining promoter activity (Fig. 2 and 3). Although transcription of yeast mtDNA also initiates within a highly conserved consensus sequence (4, 5, 18, 32), mammalian mtDNA promoters require extensive upstream sequences for full promoter activity (14, 15, 23).

The deletion mutagenesis results in Fig. 2 and 3 provide some evidence for sequences that modulate the activity of *X. laevis* mitochondrial promoters. For the leftward deletions shown in Fig. 2, the intensity of the HSP 1 transcript relative to that of the LSP 1 transcript appeared to increase as the deletion approached the core promoter sequences. The autoradiogram shown in Fig. 2 was scanned with a densitometer to aid in quantitation of promoter strength for these deletions. The resulting quantitative estimates of promoter

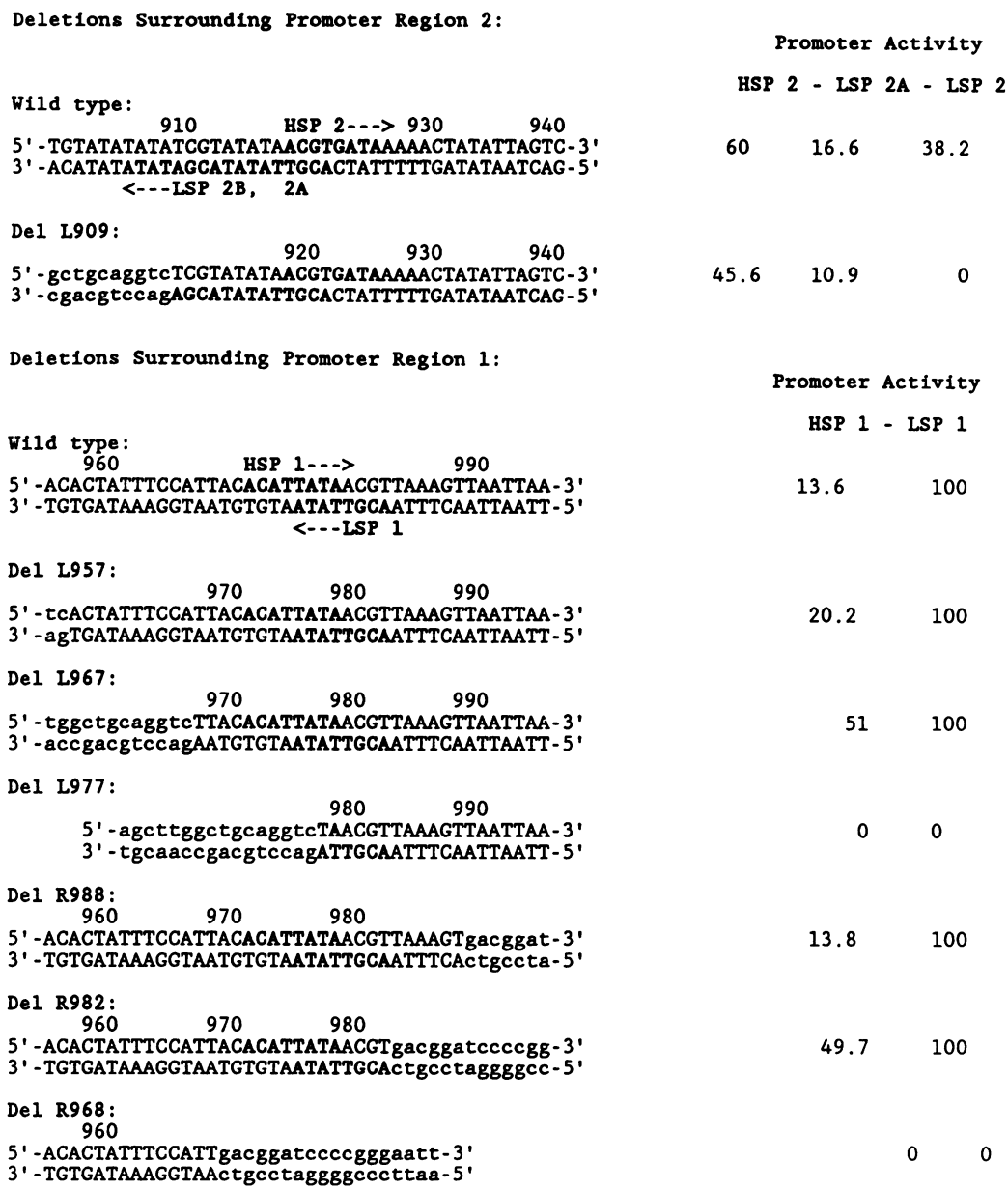
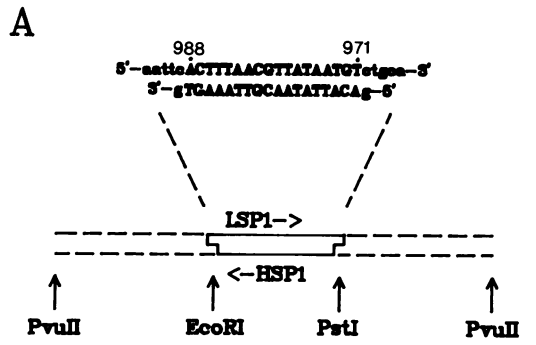


FIG. 4. Nucleotide sequences and quantitative promoter activities for selected deletion (Del) mutants. Nucleotide sequences surrounding the endpoints of critical deletions are shown with uppercase letters denoting mitochondrial sequences and lowercase letters denoting residues derived from linker and plasmid sequences. Promoter activity was quantified by densitometry of autoradiograms and is expressed as a percentage of the activity observed for the LSP 1 promoter in each clone.

activity are tabulated in Fig. 4. This analysis confirmed that progressive deletions toward the bidirectional promoter 1 region from the left resulted in an increase in the relative strength of HSP 1 from about 14% of the activity of LSP 1 in the wild type (Fig. 2B, lane 1) to approximately 50% of the activity of LSP 1 in ΔL967 (Fig. 2B, lane 5). Similar scans of autoradiograms of rightward deletions indicated that deletion from ΔR988 to ΔR982 also increases the activity of HSP 1 relative to LSP 1 (Fig. 3B, compare lanes 2 and 3; tabulated in Fig. 4). We conclude that the sequences surrounding the core sequences of promoter region 1 act either to augment the activity of LSP 1 or to inhibit the activity of HSP 1. This phenomenon is discussed in greater detail below.

Functional bidirectional promoter contained in a cloned oligonucleotide. The analysis of unidirectional deletion mutants has confirmed that the major determinant of promoter activity is an octanucleotide consensus sequence, while flanking sequences play a relatively minor role to modulate promoter strength. Thus, a short sequence encompassing a bidirectional promoter might be sufficient to direct transcription initiation. To test this hypothesis, two complementary oligonucleotides containing sequences found at the bidirectional promoter 1 were synthesized chemically, hybridized, and cloned in a plasmid vector (Fig. 5A). A *Pvu*II fragment containing this short oligonucleotide supported the synthesis of RNAs 240 and 132 nucleotides in length, consistent with active LSP 1 and HSP 1 promoters, respectively. We conclude that a short sequence encompassing the core promoter sequences from region 1 is sufficient to support bidirectional transcription initiation. However, as noted below, this syn-



B

POINT MUTATIONS IN THE SYNTHETIC BIDIRECTIONAL PROMOTER

MUTANT POSN.	HSP STRAND	LSP STRAND
	971 ... 988	988 ... 971
WT	gACATTATAacgttaaagtg	cactttaACGTTATAatgtc
973	---t----------	----------a---
973,974	---ta---------	---------ta---
975	-----g--------	--------c-----
976	------c-------	-------g------
977	-------g------	------c-------
978	--------c-----	-----g--------
979	---------c----	----g---------
980	----------g---	---c----------
981	----------a---	---t----------
982	----------g---	---c----------
983	----------a---	---t----------
983,984	----------at--	--at----------

FIG. 5. Cloning and mutagenesis of a synthetic bidirectional promoter. (A) Orientation of the wild-type oligonucleotide pair representing mtDNA sequences 971 to 988 as cloned between the *Eco*RI and *Pst*I sites of plasmid pBS−. Transcription experiments employed *Pvu*II digests of plasmids bearing point mutations within this synthetic promoter. (B) Sequences of the point mutants illustrating the position of the base changes with respect to the LSP and HSP sequences. The mutants are named as indicated on the left according to the position that the base pair change would occupy in the reference mtDNA sequence (11). WT, Wild type.

thetic promoter does not appear to have full activity for HSP 1.

The in vitro template activity of the synthetic promoter enabled us to generate a collection of point mutations within the bidirectional promoter (Fig. 5B). This collection contains mutations at each position within LSP 1. However, because the oligonucleotide sequence employed does not extend very far to the 5′ side of HSP 1, mutations were not obtained at the 5′ end of HSP 1. Most of the changes programmed were transversion mutations. The analysis of the effects of these mutations is complex, since any of these base pair changes might affect either or both of the two promoters. The consensus sequences for LSP 1 and HSP 1 overlap at the central TATA sequence. Base pair changes to the left or right of this overlap might be expected to affect only one of the promoters. If the two consensus sequences could function completely independently, we might expect that transcription would reflect a competition between these promoters for the transcription factor or the RNA polymerase or both.

All the point mutations in the synthetic promoter were transcribed along with a *Pvu*II digest of ΔL945 as an internal control in the reconstituted system containing the mtRNA polymerase and the transcription factor. An autoradiogram

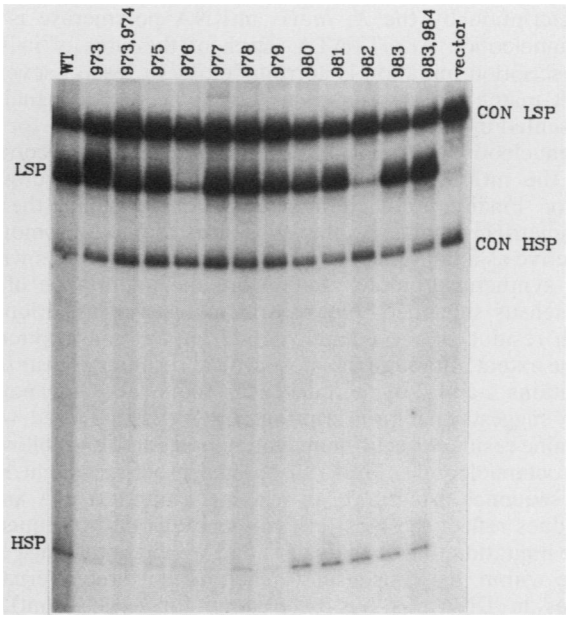


FIG. 6. Template activity of synthetic promoters bearing point mutations. Samples (0.2 μg) of *Pvu*II digests of pBS− clones bearing either wild-type or mutagenized copies of the synthetic promoter were mixed with 0.1 μg of *Pvu*II-digested pX1m ΔL945 as an internal control for template activity. The mixed templates were transcribed in vitro with the mtRNA polymerase and transcription factor as described in Materials and Methods. The ^{32}P-labeled RNAs produced were fractionated on a polyacrylamide-urea gel and detected by autoradiography of the dried gel. The individual lanes are labeled by the location of the point mutation in the synthetic promoter (Fig. 5). The lanes labeled WT and vector display transcripts resulting from template mixtures containing the wild-type promoter sequence and the pBS- vector, respectively. The labels on the left indicate the positions of the LSP and HSP transcripts of the synthetic promoter. The labels on the right indicate the positions of the LSP and HSP transcripts of the internal control template.

of the resulting transcripts is shown in Fig. 6. We will first consider the results with respect to the more active promoter, LSP 1. For this promoter, the consensus sequence, ACGTTATA, represents mtDNA heavy-strand residues 982 through 975. The data in Fig. 6 clearly indicate that several of the single base pair changes significantly reduced the activity of LSP 1. However, some residues of the octanucleotide consensus appeared to be more critical than others. For example, mutations at position 982 or 976, residues AcgttaTa of the consensus sequence, essentially inactivated the promoter. Changes at other residues in the consensus and in the adenine residues immediately preceding or following the consensus caused less remarkable decreases in transcription efficiency.

The analysis of the effects of point mutations on HSP 1 is complex. The naturally occurring HSP 1 is a relatively weak promoter (Fig. 4). The wild-type oligonucleotide copy of HSP 1 is an even weaker template than the HSP 1 surrounded by other mtDNA sequences. Changes at several residues within the consensus sequence seriously impaired HSP 1 activity (Fig. 6). It would appear that a naturally weaker promoter is more easily inactivated by single base changes.

DISCUSSION

Transcription initiation requires core consensus sequence. The predominant sequence required to promote specific

transcription by the *X. laevis* mtRNA polymerase is the octanucleotide ACGTTATA. Each of the sites of in vivo transcription initiation is surrounded by at least a seven of eight match to this consensus (10). The deletion analysis presented in Fig. 2 and 3 shows a clear requirement for this octanucleotide for in vitro transcription in reactions containing the mtRNA polymerase and a specific transcription factor. Finally, a cloned oligonucleotide containing the two symmetrical matches to this consensus found at promoter 1 is active as a bidirectional promoter. Point mutations within this synthetic promoter underscore the importance of the consensus sequence. Figure 6 indicates that mutations at each residue of the octanucleotide impair transcription to some extent, although the most critical residues appear to be positions 1 and 7 of the consensus sequence. Our analysis also suggests that transcription activity is increased when adenine residues occur immediately preceding and following the octanucleotide. Thus, an optimal promoter might have the sequence aAcgttaTa, in which the uppercase A and T residues reflect the most critical positions in the sequence. One limitation to this analysis is that we did not change each base within this consensus to each of the three alternative bases in DNA, as has been done for a yeast mtDNA promoter (5).

The consensus sequence is found at five sites within a 100-bp region of the *X. laevis* mtDNA. Two other matches to the consensus sequence are also found near the active promoters. These sequences, ACGTTAAA, just downstream from HSP 1, and ATGTTATA, upstream from HSP 2, are not active as promoters in vitro, presumably because the base changes with respect to the usual consensus sequence are not tolerated. The functional promoter sequences are clustered at two sites separated by about 60 bp. This arrangement of overlapping core promoters with dyad symmetry is quite unusual. While complex procaryotic promoters can have overlapping control sequences (28), these examples do not involve such a simple consensus sequence. There does appear to be some purpose to this clustering of promoters. Some of the results obtained with point mutagenesis shown in Fig. 6 are consistent with the possibility that proteins interact cooperatively at adjacent or overlapping promoters. The *Xenopus* mtRNA polymerase requires an accessory factor for specific transcription (7). However, we have not yet determined details of protein-DNA interactions at a bidirectional promoter. The collections of deletion mutations and point mutations described here should facilitate efforts to map the interactions of the transcriptional machinery with the DNA.

Modulation of promoter activity by nucleotide sequences surrounding the octanucleotide consensus. Our mutagenesis results showed that promoter activity requires a core consensus sequence and that a synthetic oligonucleotide with the correct dyad symmetry is sufficient to serve as a bidirectional promoter. There are at least two reasons to suspect that the octanucleotide consensus sequence is not the sole determinant of *X. laevis* mitochondrial promoter activity. First, as discussed above, the unusual bidirectional arrangement of *X. laevis* promoters may reflect cooperative interactions between promoters. Second, the fact that the five *X. laevis* mitochondrial promoters are not equally active suggests that additional sequences influence promoter activity. The same sequence that serves as a strong promoter at LSP 1 is found for a weaker promoter at LSP 2A. Similarly, both HSP 2 and LSP 2B appear to be stronger promoters than HSP 1, although neither contains an exact match to the consensus sequence (Fig. 4). Our deletion mutagenesis results suggest that sequences surrounding promoter region 1 somehow augment the efficiency of LSP 1 transcription. Deletion of sequences from either the left or the right increased the activity of HSP 1 relative to LSP 1 (Fig. 4). Deletion from the 3′ side toward LSP 1 also appeared to induce variability on the selection of start sites, as shown by the heterogeneous length of runoff transcripts in lanes 3 to 5 of Fig. 2. We do not know the basis for these flanking sequence effects. The sequences adjacent to the *X. laevis* mtDNA promoters show a number of unusual features. There are runs of adenine residues similar to those shown in other systems to induce DNA bending (26, 30, 40) following nearly every promoter. In addition, the sequence flanking the 3′ side of LSP 1 that appears to augment LSP 1 promoter activity contains two direct repeats of the sequence TTTC-CAPyTA separated by 20 bp. However, none of these sequences bears a significant relationship to upstream elements of human or mouse mtDNA promoters (14, 15, 23). Additional experiments will be necessary to define these modulatory sequences in greater detail.

Do promoters occur in other regions of the mtDNA genome? All the promoters described to date for human, mouse, and *Xenopus* mtDNAs have been identified within a noncoding region adjacent to the D loop at the origin of replication. It is conceivable that promoters that are weakly transcribed or that yield RNAs that are rapidly processed would not have been detected in our experiments to map in vivo transcription start sites (10). Since our mutagenesis results indicate that a short match to a consensus sequence is necessary and sufficient for promoter activity, it is appropriate to ask whether this sequence occurs in other regions of the mtDNA genome.

We performed computer searches for matches to the consensus sequence at other sites within the *X. laevis* mtDNA sequence. In this analysis, we used the complete genome sequence published by Roe et al. (29) and the D-loop region sequences published by us (11) and by Dunon-Bluteau et al. (21). In analyzing the output of this search, it is important to recall that the activity of a potential promoter would be influenced by clustering of promoter sequences, adenine residues immediately surrounding the octanucleotide, and additional flanking sequences that are not yet clearly defined. The sequences ACATTATA or ACGT-TATA were found at three locations. However, only one of these sites has a consensus sequence flanked by adenine residues, which appears to correlate with increased promoter activity (Fig. 6). This site is particularly interesting, since it is located adjacent to a cluster of tRNA genes surrounding the origin of light-strand DNA replication. Further experiments will be required to determine whether this potential promoter is functional in vivo or in vitro.

The five functional promoters studied in this paper include three examples each with one mismatch with respect to the consensus. Therefore, computer search conditions were set to permit one mismatch to the octanucleotide ACGTTATA. Sequences matching at seven positions of the consensus were found at several positions of the genome. A complete analysis of these sequences is beyond the scope of this paper. None of these matches were clustered so as to encode bidirectional promoters. Most of these potential promoters contain base changes that would be predicted to be deleterious based on the results shown in Fig. 6. Although we have obtained no evidence to suggest in vivo initiation at these sites (10), we cannot completely rule out transcription from these promoters. DNA fragments containing these matches have not been tested for promoter activity in vitro. How-

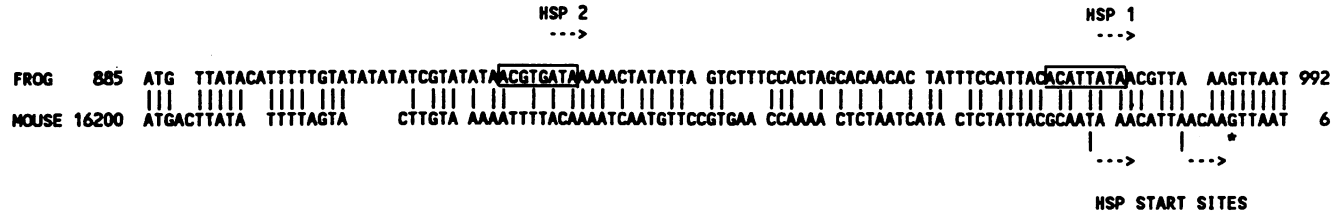


FIG. 7. Comparison of *X. laevis* promoter region with the corresponding sequences from mouse mtDNA. Residues 885 to 992 of the light strand of *X. laevis* mtDNA are shown on the top line. The HSP promoter sequences are shaded and labeled, with an arrow indicating the direction of transcription. Residues 16200 through 16295 and 1 to 6 of the light strand of mouse mtDNA (3) are shown with arrows indicating the sites of heavy-strand transcription initiation (15). Vertical dashes between the sequences indicate sequence matches.

ever, we consider it unlikely that these sequences related to the core promoters would be functional. Finally, although there is evidence for a weak transcriptional promoter at the $tRNA^{Phe}$-12 S RNA gene border in human mtDNA (27, 39), the corresponding site of *X. laevis* mtDNA does not contain a match to the consensus sequence and we found no evidence for promoter activity in this region of *X. laevis* mtDNA.

Comparison with other mitochondrial promoters. On a superficial level, the predominant influence of a short core promoter sequence for *X. laevis* mtDNA bears some resemblance to the situation for yeast mtDNA, in which transcription also initiates within a short, highly conserved sequence (4, 5, 18, 32–34). However, the two consensus sequences are dissimilar apart from a general A+T-rich character and yeast promoters are not arranged in symmetrical pairs.

While the yeast mtDNA genome contains a relatively large number of separate transcription units, vertebrate mitochondrial genomes employ only a few promoters located between the gene for $tRNA^{Phe}$ and the 5′ end of the D loop. In human, mouse, and *Xenopus* mtDNAs, heavy-strand and light-strand promoters are separated by about 150 bp or less. In each case, the light-strand promoters are positioned so as to be capable of priming D-loop-strand DNA synthesis (13, 16). The most striking distinction between the *X. laevis* promoters and mammalian promoters has been noted above, namely, that the mammalian promoters rely heavily on upstream sequence elements (13, 14, 23). It should be remembered that these upstream elements of mammalian mitochondrial promoters are principally located in the short stretch of DNA separating the two promoters. The *X. laevis* promoters are also distinguished by the fact that they are transcribed at comparable rates in both directions. Even though there is some bidirectional character to human mitochondrial promoters (17), there is a strong preference for transcription in one of the two directions.

Although no similarity between the different vertebrate promoter sequences was evident to the eye, we undertook a series of computerized comparisons of 200-bp segments of the DNA surrounding each of the *X. laevis*, human, and mouse promoters. These 200-bp sequences were located immediately upstream of $tRNA^{Phe}$. The "homology" and "matrix" comparison algorithms of the Microgenie (Beckman) programs were run under a variety of search conditions. As expected, the computer identified a larger number of matches of the *X. laevis* sequence to mouse than to human mtDNA. However, we were surprised to note that more matches were identified in the frog-to-mouse comparison than in the mouse-to-human comparison. One of the most striking matches was a 25 of 32 match between the promoter 1 region of *X. laevis* mtDNA and the sequence surrounding the sites of initiation of heavy-strand transcription for mouse mtDNA. This sequence similarity, which is not found in the corresponding location of human mtDNA, can be extended to a 66% match to 108 residues surrounding the *X. laevis* promoters. Most significantly, the two proposed sites for transcription initiation from the murine HSP are six of eight and seven of eight matches to the *X. laevis* consensus. The sequence comparison is shown in Fig. 7. However, the meaning of this sequence similarity is uncertain since Chang and Clayton (15) have suggested that the sequences surrounding the transcription start sites for the mouse HSP are not required for initiation. Although the overall sequence relationship is likely to be a vestige of the common ancestry of vertebrate mtDNA genomes, its significance relative to the contemporary mechanism of transcription is not clear. A detailed comparison of the protein-nucleic acid interactions involved in transcription initiation in the two systems should be particularly interesting.

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