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Minor Transcription Initiation Events Indicate that Both Human Mitochondrial Promoters Function Bidirectionally

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Human mitochondrial DNA is transcribed from two distinct, strand-specific promoters located in the displacement loop region of the genome. The transcriptional control sequences identified by deletion mapping and site-directed mutagenesis studies span short regions surrounding the initiation sites and bear no obvious sequence homology to any nuclear or procaryotic promoters. In vitro transcription analyses also revealed several minor initiation sites that are characterized by a pyrimidine-rich region followed by a purine-rich region, a feature that is shared by the two major promoters. In this paper, we report a new class of minor promoters in human mitochondrial DNA. These minor promoters were localized to the same duplex DNA sequences that direct major transcriptional events, but they had transcriptional polarity opposite to that of the major promoters. Furthermore, nucleotide changes that affected the major form of transcription similarly affected transcription in the opposite direction. For one of these minor promoters, a corresponding in vivo RNA species initiating from the same site was identified. These observations indicate that the major transcriptional promoters in human mitochondria can function bidirectionally both in vivo and in vitro.

The major noncoding region in animal mitochondrial DNA (mtDNA) is the displacement loop (D loop) region containing the origin of heavy (H)-strand DNA replication and two transcriptional promoters, one for each strand of the genome (see reference 10 for a review). These major promoters, the heavy-strand promoter (HSP) and light-strand promoter (LSP) (7), direct synthesis of polycistronic transcripts and are likely responsible for expression of all genes encoded by mtDNA (see reference 11 for a review). In addition, the LSP is involved in the priming of H-strand DNA synthesis (8, 9).

Using in vitro transcription assays, we have previously identified the control sequences for both promoters. Deletion mapping studies have localized the HSP to −16 to +7 and the LSP to −28 to +16 of their respective transcriptional start sites (7). Although these promoters are located within 150 base pairs (bp) of each other, the regulatory sequences do not overlap, and each can function in vitro in the absence of the other. The sequence requirements of each promoter have been further characterized by site-directed mutagenesis experiments (14), the results of which suggest that the minimal promoter sequences in human mitochondria may consist of a cystosine-rich region followed by an adenine-rich region. Consistent with this idea is the observation that many pyrimidine-rich regions followed by a few adenine residues can support in vitro transcription (7). These minor promoters are less efficient than the two major promoters, indicating a role in efficient gene expression for nucleotide sequences surrounding the major promoters.

We report here minor transcripts initiating from the major transcriptional start sites but proceeding in opposite directions. To characterize the required control sequences for these events, we studied the in vitro expression of a series of deletion clones and base-substituted mutants. Furthermore, we identified in vivo RNA species that initiate from one of these minor promoters. These data demonstrate that the major promoters in human mitochondria can effect bidirectional transcription both in vivo and in vitro.

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MATERIALS AND METHODS

RNA filter hybridization. Nucleic acids from human KB cell mitochondria isolated by discontinuous sucrose gradient centrifugation were purified with hot-phenol extraction as previously described (27). Isolated mitochondrial RNA (mtRNA; 10 μg) was fractionated by 1.4% agarose–1 M formaldehyde gel electrophoresis (16), except that the running buffer contained 20 mM sodium borate (pH 8.3), 0.2 mM EDTA, and 1 M formaldehyde. Following electrophoresis, the mtRNA was transferred to a nitrocellulose filter and hybridized with RNA probe labeled with [α-^{32}P]GMP (Amersham Corp.).

The recombinant plasmid used as the template for RNA probe synthesis was constructed by introducing a *Bam*HI-*Eco*RI fragment of a deletion clone containing mtDNA sequences (positions 408 to 739) into a pSP64 vector (20). The *Fnu*4H1 site between the bacteriophage SP6 promoter and the mtDNA insert was removed by digestion with *Hin*dIII and *Sal*I; the staggered ends were repaired with *Escherichia coli* DNA polymerase (large fragment) (New England BioLabs, Inc.) and religated with T4 DNA ligase (New England BioLabs). This modification was necessary to linearize the plasmid with a second *Fnu*4H1 site within the mtDNA insert. In vitro transcription with SP6 RNA polymerase (Promega Biotec) and filter hybridization with the RNA probe was carried out as previously described (4, 9).

In vitro transcription. Constructions of clones containing mtDNA template sequences altered by nuclease BAL 31 deletion and sodium bisulfite chemical mutagenesis have been described before, and most clones have been characterized previously (7, 14).

Human mtRNA polymerase was purified by heparin-Sepharose column fractionation as previously described (29). A standard transcription reaction (50 μl) contained 20 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg of bovine serum albumin per ml, 0.2 mM each of four ribonucleoside triphosphates, and 1 μg of DNA. Following addition of 10 μg of mtRNA polymerase fraction, the reaction mixture was incubated for 30 min at 30°C. The reaction was terminated by addition of sodium dodecyl

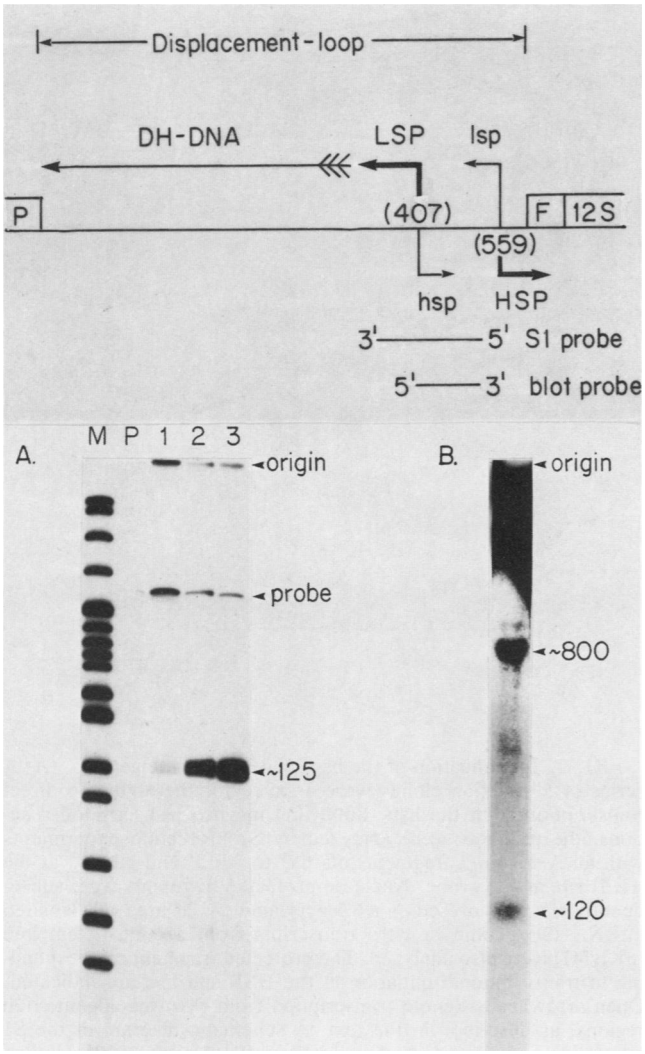

FIG. 1. (Top) Organization of human mtDNA and in vivo transcriptional analyses of the spacer region between the two major promoters. The D loop is shown, including the 5′ and 3′ termini of D loop H-strand DNA (DH-DNA) and the structural genes for $tRNA^{Pro}$ (P), $tRNA^{Phe}$ (F), and 12S rRNA (12S). The relative locations of the LSP, HSP, hsp, and lsp and their respective initiation sites at nts 407 and 559 are also shown. The polarities and positions of the DNA probe used in the S1 protection analysis and the RNA probe used in the filter blot are also indicated. (A) S1 protection analysis of upstream H-strand transcripts. The S1 probe was generated from a 263-bp *Fnu*4H1 fragment by 5′ end labeling and isolating the H strand (nts 526 [5′] to 263 [3′]). (The *Fnu*4H1 site at nt 260 is a polymorph not present in the human mtDNA sequence reported by Anderson et al. [1].) Lanes: M, *Hpa*II digests of pBR322; P, probe only; 1, in vitro transcripts from pKB741; 2, 10 μg of mtRNA; 3, 20 μg of mtRNA. The protected fragments were electrophoresed on a 6% acrylamide–7 M urea gel. (B) Northern blot analysis of upstream L-strand transcripts. A 118-nt [α-^{32}P]GMP-labeled RNA (nts 408 [5′] to 526 [3′]) synthesized in vitro from a bacteriophage SP6 promoter was used as the probe. The sizes of two major RNA species detected with this probe are shown next to the lane. The high-molecular-weight species hybridizing to the probe were due to contaminating mtDNA present in total mtRNA preparations isolated by phenol-chloroform extraction. The nt numbering is according to Anderson et al. (1).

sulfate to a final concentration of 0.2%, followed by phenol-chloroform (1:1) extraction. RNA was recovered by ethanol precipitation and treated with DNase I (RNase free; Miles Laboratories, Inc.) as previously described (7).

Nuclease S1 protection. 5′ termini of RNA were identified by a nuclease S1 protection protocol (2). Probes were generated by isolating restriction fragments 5′ end labeled with T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P]ATP (Amersham) as described by Maxam and Gilbert (17). The precise location of each probe is described in the corresponding figure legend. Hybridization was carried out in 30 μl of 80% formamide–0.4 M NaCl–50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (pH 6.4)–1 mM EDTA at 40°C for 3 h. The hybridization mixture was subsequently digested with 15,000 U of nuclease S1 (Boehringer Mannheim Biochemicals) per ml. Protected fragments were analyzed by electrophoresis in 6% acrylamide–7 M urea gels.

Primer extension analyses. Primer extension analysis was carried out as previously described (7). The primer used for the characterization of transcripts from 5′ deletion clones (see Fig. 4A) was generated from a deletion clone containing mtDNA sequences 460 to 739. The plasmid DNA was restricted with *Bam*HI, 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and subsequently cleaved with *Fok*I (New England BioLabs). The resulting 36-nucleotide (nt), single-strand fragment (mtDNA sequences 460 [5′] to 488 [3′]) was isolated by denaturing-gel electrophoresis (17). The 5′ end of the primer contains an additional 7 nts of linker DNA sequences introduced during the cloning procedures. These additional nts did not interfere during the primer hybridization or extension procedures.

The primer used (see Fig. 4B) was generated from a *Bam*HI-*Hha*I restriction fragment of pBR322. pBR322 was digested with *Bam*HI, 3′ end labeled with *E. coli* DNA polymerase (large fragment) and [α-^{32}P]dGTP (Amersham), and subsequently cleaved with *Hha*I. The single-strand DNA containing pBR322 sequences (26) 414 (5′) to 379 (3′) was isolated and used as the primer.

RESULTS

Both strands in the region between the two major promoters are transcribed. The 5′ region of the D loop and the initiation sites of the two major promoters are shown in Fig. 1. The HSP and LSP are located close to each other, with only 150 bp separating the respective transcriptional start sites within them. This 150-bp region contains most or all of the transcriptional regulatory sequences for the HSP and LSP (7, 14) and does not encode any known functional genes. Since primary transcription from the major promoters proceeds in opposite directions, the 150-bp intervening region will never be transcribed from the HSP or LSP, unless RNA polymerase makes a complete round of transcription of the entire 16-kilobase circular genome. To ascertain whether this upstream region is transcribed, we analyzed mtRNA with nuclease S1 protection and Northern blot experiments. To map H-strand transcripts in this region, we carried out nuclease S1 protection with a 5′ end-labeled, single-strand DNA probe. The 5′ end of the probe is located upstream from the major H-strand promoter, and therefore transcripts from the HSP were not detected in this assay. The results shown in Fig. 1A demonstrated a protected fragment of ~125 nt in length when either mtRNA (lanes 2 and 3) or in vitro transcribed RNA (lane 1) was used. The 5′ end positions of the RNA species mapped approximately to nt 407. Interestingly, major transcription of the complementary

strand in the opposite direction from the LSP also initiates at nt 407 (7).

In vivo transcripts mapping upstream from the LSP were characterized by Northern blot analysis with a radiolabeled RNA probe synthesized by SP6 RNA polymerase. This ~120-nt RNA probe is uniquely complementary to the H strand of the 150-bp region and therefore hybridizes only to L-strand transcripts containing this 150-bp sequence. The analysis showed two RNA species of ~800 and ~120 nt in length (Fig. 1B). Because of the low abundance of these transcripts, the exact nt positions of 5′ termini of the RNA species could not be determined. The presence of L-strand transcripts containing the sequence of the 150-bp region can therefore be accounted for either by a complete round of transcription of the entire mitochondrial genome from the LSP or, alternatively, by a transcriptional event from a minor LSP (lsp; see below). In summary, the above experiments demonstrated that both strands of the 150-bp region between the two major promoters are transcribed and mapped the 5′ termini of the upstream H-strand transcript at nt 407.

Deletion mutant analyses of an upstream hsp. An upstream H-strand transcript was seen in the above S1 protection analysis when the products of in vitro transcription from a template containing an mtDNA insert were analyzed (Fig. 1A, lane 1). The 5′ termini of these in vitro transcripts mapped around nt 407. These data suggested a minor transcriptional event initiating around nt 407. To delineate the control sequences for this putative minor HSP (hsp) and to eliminate the possibility of a posttranscriptional processing event generating a 5′ end at nt 407, we analyzed the products of in vitro transcription with a series of truncated DNA templates. We subsequently determined the 5′ termini of in vitro transcripts by S1 protection experiments by using as the probe an *Ava*II-*Sac*I restriction fragment uniquely 5′ end labeled at the *Ava*II site.

A protected fragment of ~250 nt corresponding to the upstream H-strand transcript was seen when pKB741 or clones deleted to −63, −41, and −15 nts relative to the minor initiation site at nt 407 were used as the templates (Fig. 2A). Further deletion of mtDNA sequences 21 nts beyond the initiation site (+21) abolished the signal. Therefore, the 5′ boundary of the hsp is located within 15 nts upstream from the transcriptional initiation site at nt 407. Furthermore, the fact that the ~250-nt fragment was seen even when most 5′ sequences of the template DNA were deleted suggests that the 5′ end of RNA at nt 407 is not likely generated by posttranscriptional processing of longer RNA species from the upstream region (see Fig. 3). The strongly protected fragment of ~100 nt seen in most lanes corresponds to transcription from the major HSP. Two other minor species of ~270 and ~170 nts (open arrowheads) seen in some of the lanes mapped to a cytosine-rich region followed by one or two adenine residues in the template. Such regions tend to serve as minor promoters in in vitro transcription assays (7). A series of progressively shortened fragments, seen in lanes +21, +59, +136, and +154, represent readthrough transcripts initiating from the pBR322 vector sequences resected to more homogeneously sized fragments upon nuclease S1 treatment by virtue of noncomplementarity between the mtDNA probe and upstream pBR322 sequences.

The 3′ boundary of the hsp was similarly established by analyzing in vitro transcription products from truncated templates by an S1 protection experiment. The RNA synthesized from the 3′ deletion clones cannot be assayed with a common S1 probe because the templates contain deletions within the transcribed region and therefore transcripts from each 3′ deletion clone will be different in sequence. Initially, primer extension experiments, which do not require a significant stretch of sequence complementarity between the primer and the RNA, were performed but were unsuccessful owing to the low abundance of transcripts and inefficient elongation of the primer. Therefore, we isolated individual S1 probes for four different 3′ deletion clones to identify the 3′ boundary of the hsp. When in vitro transcripts from the templates deleted to +104, +56, and +29 (initiation site at +1) were analyzed with corresponding S1 probes, protected fragments of ~380, ~340, and ~290 nts were seen (Fig. 2B).

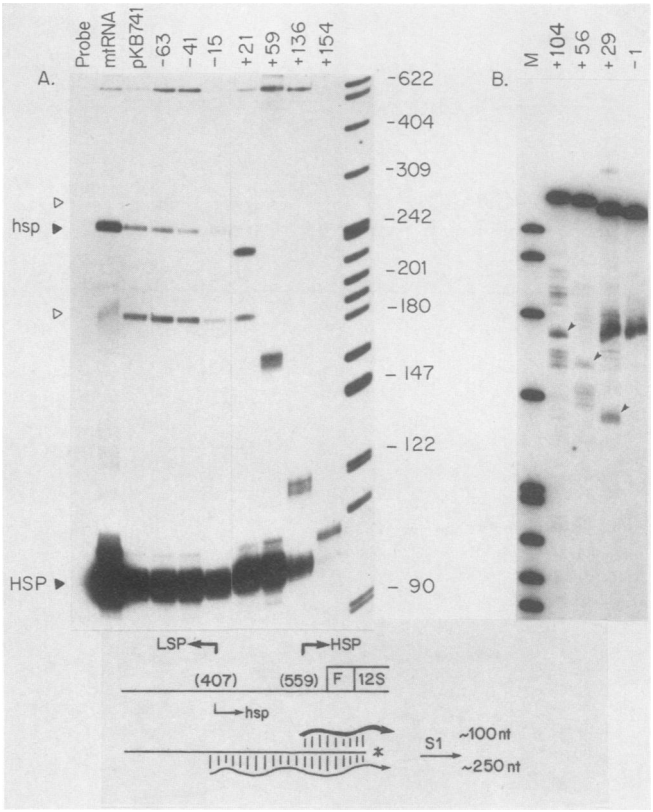

FIG. 2. Identification of the hsp by deletion mutagenesis. (A) A series of 5′ deletion clones were assayed for the ability to direct transcription from the hsp. Following in vitro transcription reactions, the products were analyzed by S1 protection experiments with an *Ava*II-*Sac*I fragment (nts 657 to 37) 5′ end labeled at the *Ava*II site as the probe. Nuclease-protected fragments were subsequently electrophoresed on a 6% acrylamide–7 M urea gel. Isolated mtRNA (2 μg) and in vitro transcripts from a control template (pKB741) were also analyzed. The protected fragments corresponding to transcriptional initiation at the HSP and hsp are indicated. Open arrowheads denote transcription from cytosine-adenine-rich regions, as described in the text. A schematic diagram of the S1 protection experiments is shown below. (B) A series of 3′ deletion clones were assayed for the ability to direct transcription from the hsp. S1 probes were generated by isolating an *Sal*I-*Eco*RI fragment (5′ end labeled at the *Sal*I site) from the deletion clone used in each transcription reaction. The protected fragments of ~380, ~340, and ~290 nts, corresponding to transcripts from the hsp, are indicated by arrowheads. The minor, distorted bands in lanes +29 and −1 are electrophoretic artifacts. In both A and B, lanes are labeled with the position of the last nts retained in each deletion clone, as numbered relative to the initiation site at nt 407 (+1). The size markers are based on *Hpa*II digests of pBR322.

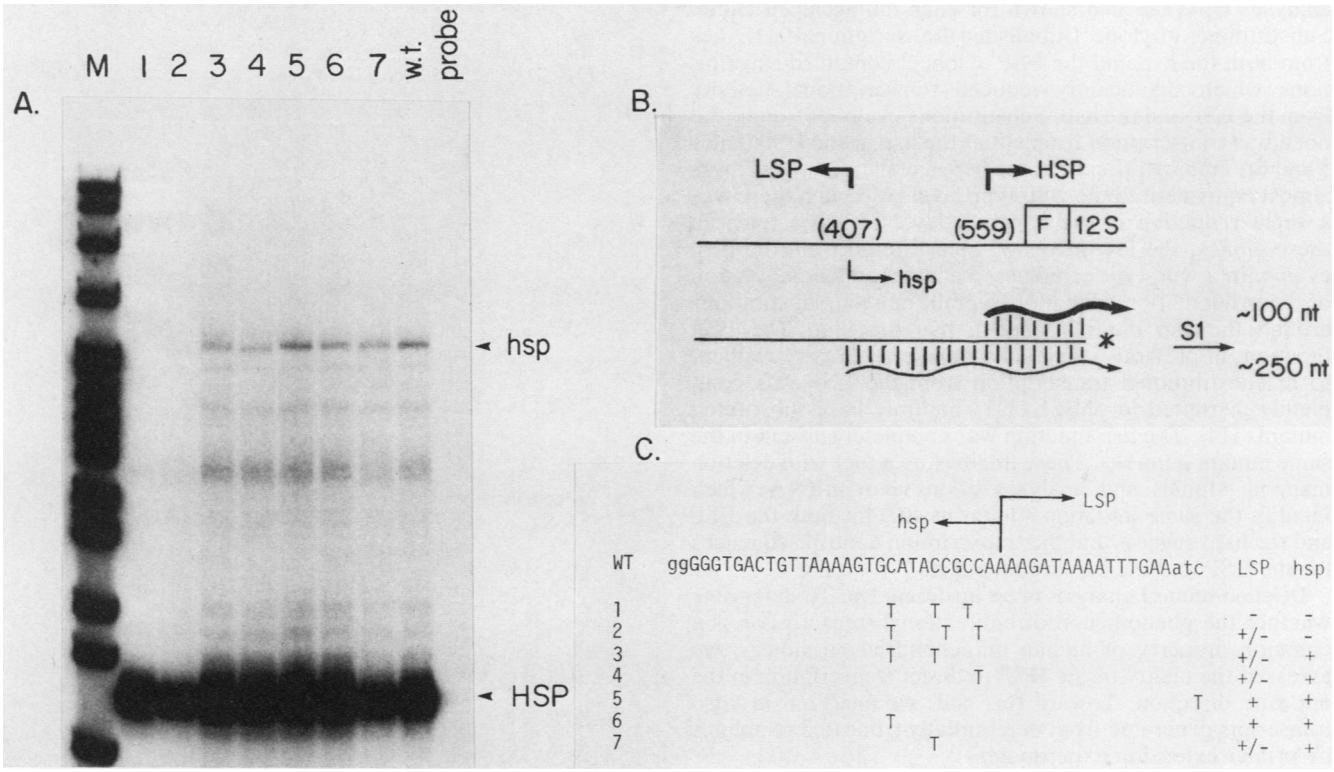

FIG. 3. Characterization by site-specific mutagenesis of the nt sequence required for hsp function. (A) In vitro transcripts from wild-type template (lane w.t.) and mutagenized clones (lanes 1 to 7) were analyzed by S1 protection. The positions of nuclease-protected fragments corresponding to transcription from the hsp and from the HSP are indicated. In lane M, *Hpa*II digests of pBR322 were electrophoresed as size standards. (B) A region of the D loop, with positions and transcriptional polarities of the HSP and LSP (→) and the hsp (⇝), is shown. The location of the S1 probe (an *Ava*II-*Eco*RI restriction fragment; nts 657 to 1) is also indicated. Transcripts from the HSP (〰) and hsp (⇝) resulted in S1 nuclease-protected fragments of ~100 and ~250 nts, respectively. (C) The sequence of nts 389 to 437 encompassing the hsp (uppercase letters) and base substitutions present in each mutant clone are shown. The sequence is that of the coding H strand. Mutant clones are designated according to the lane numbers in A. For each clone, the levels of transcription from the hsp and LSP are indicated as follows: −, no activity or barely detectable activity; +/−, reduced activity; +, wild-type level of activity.

The protected fragments differed in size, since the templates were deleted within the transcribed region. The 5′ termini of corresponding transcripts all mapped around nt 407. A template further deleted to −1 failed to give any specific signal. The 3′ boundary of the hsp is, therefore, localized to within 29 nts of the transcriptional start site.

Effects of site-specific nt changes are identical on both the hsp and the LSP. Deletion mutagenesis analyses indicated that the control sequences for the minor hsp were located within a 44-bp region between nts 392 and 435 (−15 to +29), the region previously shown to contain the LSP control sequences (nts 435 to 392) which direct transcription of the opposite strand (7). There are no obvious inverted or palindromic sequences in this region that could function as two distinct promoters of similar sequence. To investigate the possibility that a single control region may function as a bidirectional promoter, we studied the effect on transcription of nt transitions in the promoter region.

A set of chemically mutagenized clones containing substitutions in a region which includes the LSP has been used to identify the nucleotides that are critical for LSP function (14). These clones also contain sequence alterations in the region required for transcription from the hsp. To define the sequence requirements for hsp function, we analyzed in vitro transcription products of these mutagenized clones by S1 protection experiments. The transcripts from some of the nt-substituted mutants contain sequence alterations and therefore are not perfectly complementary to the S1 probe. However, no significant cleavage at these mismatched sites was observed upon nuclease S1 digestion, consistent with the observation that RNA-DNA heteroduplexes containing small mismatches are not normally cleaved by nuclease S1 (22; unpublished observations).

The results of experiments with in vitro transcription products from wild-type template and several mutagenized clones are shown in Fig. 3A. Protection of the 5′ end-labeled mtDNA probe from nuclease digestion (Fig. 3B) by transcripts initiating from the hsp produced fragments of ~250 nt (lane w.t.). Similarly, nuclease protection by transcripts from the HSP produced fragments ~100 nt in size. All of the mutant templates contained a wild-type HSP sequence, providing an internal control for some variation in the level of transcription in each in vitro assay. Two of the seven mutants containing G→A transitions (lanes 1 and 2) were defective in directing transcription from the hsp. Nt substitutions in clone 1 abolished transcription from the hsp, whereas those in clone 2 significantly reduced transcriptional efficiency from the hsp. Nt substitutions in the other five clones did not significantly affect hsp function (lanes 3 to 7).

The locations of G→A transitions in each mutant and the ability of each mutagenized clone to direct transcription from the hsp are summarized in Fig. 3C. The effects of base substitution on transcription in the opposite direction from the LSP, as previously determined in runoff transcriptional

analyses (14), are also shown for each mutagenized clone. Substitutions in clone 1 abolished transcriptional activities from both the hsp and the LSP. Clone 2 contained substitutions which significantly reduced transcriptional activity from the LSP and the hsp. Substitutions in clones 5 and 6 did not affect transcription from either the hsp or the LSP (lanes 5 and 6). Transcription from the hsp in clones 3, 4, and 7 was almost equivalent to the wild-type level. Although there was a slight reduction in the apparent level of hsp activity in these clones, the low levels of transcription from the hsp, even with a wild-type template, made accurate quantitation of these fluctuations difficult. Overall, most nt substitutions around the hsp did not abolish hsp function. The LSP function, in previous studies, has also proven very resilient to nt substitutions; transcription from the LSP was completely disrupted in only 1 of 13 multiply base-substituted mutants (14). The hsp function was completely absent in the same mutant (clone 1). These findings, together with deletion mapping studies and analyses of in vivo mtRNA which identify the same initiation site (at nt 407) for both the LSP and the hsp, suggest that the transcription control sequences for the LSP and the hsp are identical.

Deletion mutant analyses of an upstream lsp. To determine whether the phenomenon of bidirectional transcription is a common property of human mitochondrial promoters, we assessed the ability of the HSP to direct transcription in the opposite direction. Toward this end, we analyzed in vitro transcripts generated from differentially truncated templates by primer extension experiments.

The results of 5′ deletion analyses of the upstream lsp are shown in Fig. 4A. A 5′ end-labeled 36-nt fragment corresponding to mtDNA sequences 460 (5′) to 488 (3′) was isolated and used as the primer. The 5′ end of the probe contained an additional 7 nt of nonmitochondrial DNA sequence introduced during cloning procedures. These additional nt had no deleterious effect during hybridization or in the subsequent elongation step. An extended fragment of ~110 nt was seen when pKB741, which contains the entire 5′ region of the D loop, was used as the template for in vitro transcription. The 5′ end of the corresponding transcript mapped to nt ~559. Deletion of template sequence to −99, −61, −12, or −6 did not affect transcription from the lsp. Deletion beyond the initiation site to the +13 position of the transcribed region abolished lsp activity. Therefore the 5′ limit of the control sequences for the lsp could be assigned to within 6 nt upstream from the initiation site.

We similarly delineated the 3′ boundary of the control sequences by performing primer extension on in vitro transcripts generated from 3′ truncated templates. A primer located within the downstream pBR322 vector sequences was generated by isolating an end-labeled restriction fragment. Since the distance between the lsp initiation site and the primer is variable in 3′ truncated templates, a series of fragments of decreasing size was generated on elongation of the primer (Fig. 4B). In the first two clones, which contain an intact major LSP, elongation products of ~100 and ~50 nt resulted, corresponding to the major L-strand transcripts initiating at nt 407 (lanes +216 and +168). Also seen were longer products of ~260 and ~215 nt, respectively, the 5′ ends of which mapped to nt ~559. The difference in the sizes of these two fragments reflects the extents of 3′ deletion in the two templates, as discussed above. Subsequent 3′ deletion of template sequence to +151, +132, +94, +61, +36, or +17 did not affect upstream L-strand transcription from nt 559. The 5′ ends of the progressively shortened major elongation products mapped to approximately nt 559.

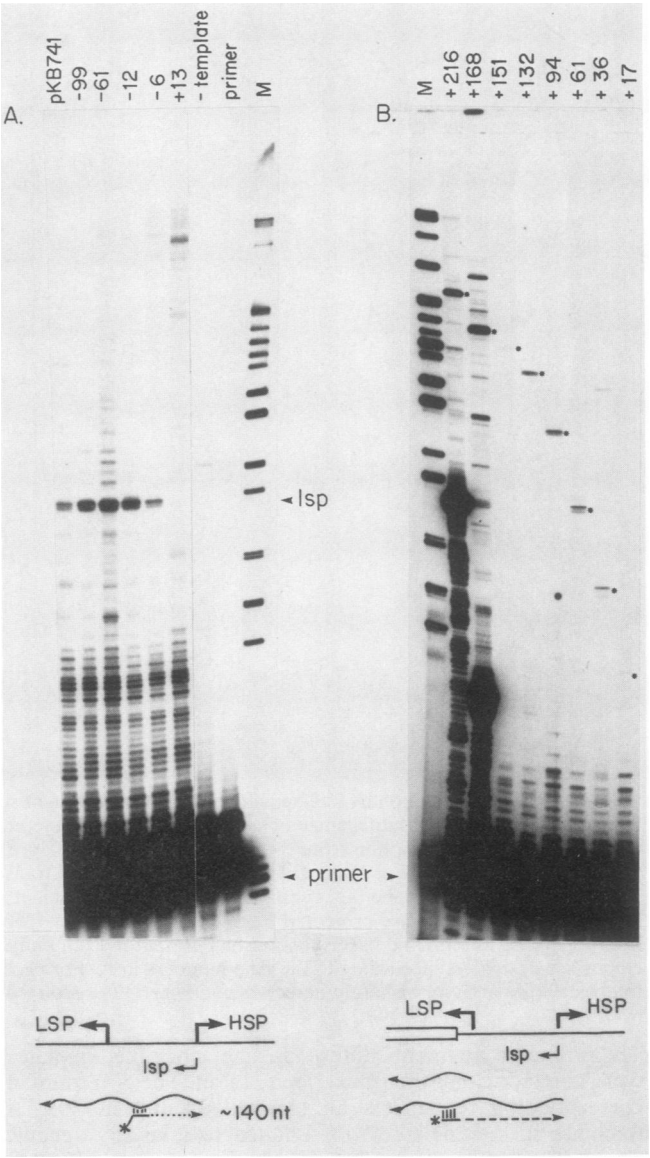

FIG. 4. Identification of the lsp by deletion mutagenesis. Series of 5′- (A) and 3′-truncated (B) clones were assayed for the ability to direct transcription from the lsp. In vitro transcription products were analyzed by the primer extension technique. The resulting extended products were electrophoresed on 8% sequencing gels. Lanes are labeled with the positions of the last nts retained, as numbered relative to the lsp initiation site at nt 559 (+1). The elongation products corresponding to transcripts from the lsp are labeled in A and denoted by dots in B. Schematics of primer extension experiments are shown below. *Hpa*II digests of pBR322 are included as size standards (lane M).

Thus, the control sequences for the lsp, defined by deletion analyses, lie within −6 to +17 of the initiation sites corresponding to mtDNA sequences 543 to 565. The same 23-bp region has previously been shown to encompass the HSP (7).

Both the HSP and the lsp respond identically to nt substitution. To investigate the possibility that identical sequences are essential for transcription from both the HSP and the lsp, we examined lsp transcriptional activities on chemically mutagenized templates. These mutants contain substitutions in the region encompassing the initiation site of transcription

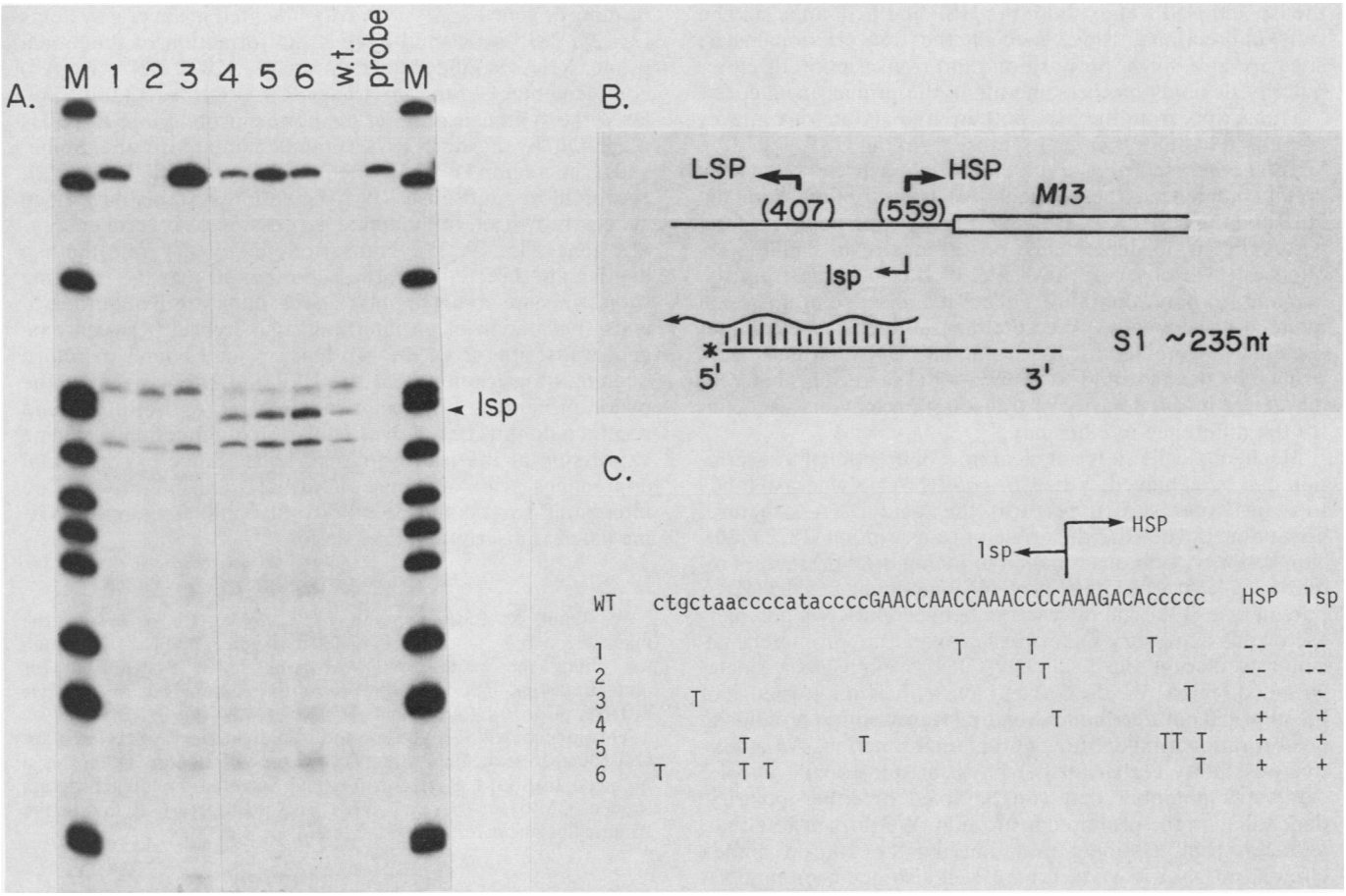

FIG. 5. Characterization by site-specific mutagenesis of the nt sequence required for lsp function. (A) S1 nuclease protection analyses of a wild-type clone (lane w.t.) and mutagenized clones (lanes 1 to 6) are shown. A *Bal*I-*Eco*RI restriction fragment (nts 323 to 739) 5′ end labeled at the *Bal*I site was used as the S1 probe. The position of a 235-nt fragment corresponding to transcription from the lsp is indicated. *Hpa*II digests of pBR322 are also shown as size standards (lanes M). (B) A region of recombinant M13 clones used as templates for in vitro transcription is diagrammed, with the position of the S1 probe shown below. The dotted line represents the 3′ region of the S1 probe containing sequences not complementary to M13 vector (see the text). (C) The sequence of nts 570 to 525, including the lsp (uppercase letters) is shown. The sequence is that of the coding L strand. The base substitutions in each mutant clone, designated by lane numbers in A, are presented below. For each clone, the levels of transcription from the lsp and HSP are indicated as follows: −−, not detectable; −, barely detectable; +, wild-type level.

from the HSP and lsp and have been tested previously for their competence to support transcription from the HSP (14).

Nuclease S1 protection analyses of in vitro transcripts generated from the mutant mtDNA templates are shown in Fig. 5A. Nuclease protection of a 5′ end-labeled mtDNA probe (Fig. 3B) by transcripts from the lsp resulted in a 235-nt fragment (Fig. 5A, lane w.t.). Of the six mutagenized clones assayed, two contained sequence substitutions which completely abolished transcription from the lsp (lanes 1 and 2), one produced greatly reduced levels of transcription (lane 3), and three exhibited normal levels of transcriptional activity from the lsp. In each lane, two additional protected fragments were observed (220 and 250 nt in length). The smaller of these fragments was due to minor transcriptional events from a cytosine-adenine-rich region. The larger fragment mapped to the position of sequence divergence between the template DNA and the radiolabeled probe (Fig. 5B). As described above, readthrough transcripts from upstream vector sequences were reduced to a fragment of discrete size on nuclease S1 digestion.

The nt changes in each mutant used to determine sequence requirements for transcription from the lsp are shown in Fig. 5C. The ability of each mutant to direct transcription from the lsp or HSP is also indicated. In mutants that had no detectable effect on HSP function, the lsp also functioned normally. Substitutions which reduced transcription from the HSP had parallel effects on lsp function. This correlation in the response of lsp and HSP activity to sequence alteration indicates that the same control sequences may direct transcription bidirectionally.

DISCUSSION

The sequences required for selective transcription from two minor promoters (hsp and lsp) were defined with templates altered by either deletion or site-specific mutagenesis. The hsp was delimited to a 44-bp region, within −15 and +29 of the transcriptional initiation site at nt 407. Similarly, the control sequence of the lsp is limited to a 23-bp region from −6 to +17 of the transcriptional initiation site at nt 559. A surprising finding is that the boundaries of the hsp and lsp coincide exactly with those of the major promoters (the LSP and HSP, respectively). Furthermore, the transcriptional initiation sites within each bidirectional promoter mapped at identical positions, nt 407 for the hsp and LSP and nt 559 for

the lsp and HSP. Thus, both the HSP and LSP appear to be truly bidirectional, since in each the transcriptional start sites are at a single bp position, and transcription of either polarity depends on the same nts in the primary sequence.

Transcripts from the hsp, both in vivo and in vitro, never amounted to more than 2% of those from the HSP (Fig. 2A). We and others have observed that the 5′ upstream sequences greatly influenced the efficiency of transcription from the HSP and the LSP (3, 7). However, the efficiencies of hsp and lsp exhibited no dependence on the upstream flanking sequences. The observed low level of transcription from the hsp and lsp may, therefore, reflect the absence of upstream modulating sequences. We note that, although the promoter sequences were identical, the actual DNA strands transcribed by the hsp and LSP were not the same. This inherent difference in the polarity of transcription may also account for the difference in efficiency.

Mechanistically, it is not clear how bidirectional transcription can be achieved. When *E. coli* RNA polymerase binds to a promoter site, it perturbs the local DNA structure, unwinding the helix at the origin of transcription (18, 24, 30). Transcription from mammalian mitochondrial promoters requires at least one other protein component besides RNA polymerase (13). The interaction between mtRNA polymerase or this accessory factor (or both) and the promoter may similarly disrupt the local DNA helix, exposing a single-stranded region. We do not yet have sufficient knowledge of the physical nature of mitochondrial transcription proteins to posit a rational model for bidirectional function. An attractive possibility is that further physical studies will reveal a symmetry potential that can be fixed in either polarity, depending on the protein constitution. We further note that, although transcriptional initiation must occur on duplex circular mtDNA in vivo, it may well also occur on mtDNA containing a triple-stranded D loop, which presents a different topological situation with regard to the physical relationship between the two potential template strands.

We do not know the role of the upstream RNAs (transcripts from the hsp and lsp). The presence of these RNAs in mtRNA isolates (Fig. 1) indicates that the upstream RNAs are not just artifacts of the in vitro transcription system. Furthermore, the LSP in mouse mitochondria also functions bidirectionally in vitro (unpublished observation). In *Xenopus laevis* mitochondria, L-strand transcription initiates from two distinct positions (D. F. Bogenhagen, personal communication). One of these sites maps very near the H-strand transcriptional start site. The transcriptional promoter sequences in higher eucaryotic mitochondria are species specific and are not conserved (7; unpublished observations). In view of such divergence in the primary sequence of the promoters, the common occurrence of bidirectional transcription in several higher eucaryotic mitochondria argues for its functional significance.

Minor initiation events in the region upstream from the cap sites have been observed in several eucaryotic genes. α-Amanitin-independent transcription from the upstream region of β-globin genes has been observed in mouse and human erythroid cells (5, 6). Recently, Farnham et al. (12) have reported the presence of opposite-strand RNA from the 5′-flanking region of the mouse dihydrofolate reductase gene. Although the function of these minor transcripts remains speculative, it is conceivable that upstream initiation may influence the expression of a downstream gene.

Transcription from the hsp and lsp produces RNAs that can hybridize with each other. A role for complementary RNA or antisense RNA in the regulation of DNA replication priming or gene expression is documented in several systems (21, 25, 28). In plasmid ColE1, the formation of functional primer RNAs is influenced by a small ~110-nt RNA (RNA I) complementary to the preprimer RNA (RNA II) (28). Similarly, the synthesis of outer membrane protein F in *E. coli* is regulated by a short RNA complementary to the Shine-Dalgarno region of outer membrane protein F RNA (21). Furthermore, antisense RNA regulation systems have been successfully used to modulate expression of several eucaryotic genes (15, 19, 23). Transcriptional events from the hsp and lsp are directed toward each other so that the resulting transcripts are complementary over the first 150 nt at their 5′ ends. Formation of an intermolecular hybrid between nascent transcripts from the hsp and lsp may hinder or retard ongoing transcription near the LSP and HSP or render the major promoters physically inaccessible or both. An end result could thus be a down-regulation of mitochondrial gene expression at the transcriptional level. Since experimental approaches with antisense RNA are feasible, it will be interesting to test the possibility of complementary RNA-mediated transcriptional regulation.

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