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Initiation of transcription from each of the two human mitochondrial promoters requires unique nucleotides at the transcriptional start sites

(displacement loop/mutagenesis/regulatory sequences/template signals)

JAMES E. HIXSON AND DAVID A. CLAYTON

Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305

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ABSTRACT **Promoters for transcriptional initiation on each strand of human mtDNA lie in close proximity in the displacement-loop region. Transcription initiates within these promoter elements, providing an opportunity to analyze the effects of nucleotide sequence changes on the efficiency and fidelity of this event within two delimited regions. Each promoter was individually altered by a site-directed mutagenesis approach and assayed *in vitro* for the ability to support transcription. The data clearly confirm the previous assignment of promoter functions for these elements and show that single nucleotide substitutions immediately upstream of the transcriptional start sites can abolish transcription. In all cases of decreased transcription, the correct site of initiation is maintained. Although the heavy-strand promoter and light-strand promoter are similar in primary sequence, they exhibit remarkably different sensitivities to base substitutions.**

Human mtDNA contains two major promoters for transcription, the heavy (H)-strand promoter (HSP) and the light (L)-strand promoter (LSP) (1). The HSP has been localized to −16 to +7 of the transcriptional start site (1, 2), and the LSP, to −28 to +16 of the transcriptional start site (1). Although these promoters are in close proximity within the displacement-loop region of the genome (3, 4), each can function, at least *in vitro*, in the absence of the other (1).

Because transcriptional initiation occurs within these short regulatory elements, it is possible to test directly the effects of sequence alterations at or near the start sites of RNA synthesis. We have used a site-directed mutagenesis approach to analyze the effects of nucleotide sequence changes in the HSP and LSP. The data show that nucleotides immediately upstream from the transcriptional start sites in the HSP and LSP are essential for transcriptional activity.

MATERIALS AND METHODS

Construction of Mutagenized Clones. mtDNA clones were constructed by insertion of the mtDNA products of *Eco*RI/*Bam*HI digestion of various recombinant pBR322 deletion clones (1) into the bacteriophage M13 vector mp8. The recombinant M13 clones used for mutagenesis of L-strand sequences containing the HSP are designated H3′Δ−47, H3′Δ−12, and H3′Δ+13 (1). Those used in H-strand sequence mutagenesis of the HSP are H5′Δ+29 and H5′Δ−60. For mutagenesis of H-strand sequences containing the LSP, recombinant M13 clones L3′Δ−58 and L3′Δ+28 were constructed. Clones L5′Δ+1 and L5′Δ−70 were used in LSP mutagenesis of L-strand sequences.

Specific regions of cloned mtDNA were exposed, as single-stranded regions, to modification by sodium bisulfite. By annealing single-stranded and *Bam*HI-linearized replicative form (RF) DNAs from the combination of clones listed above, gapped molecules were constructed in which either the HSP or LSP on each strand of mtDNA was exposed to mutagenesis. DNAs were annealed by boiling a mixture of single-stranded and RF DNA (4 μg) in 50 mM NaCl/10 mM MgCl/10 mM Tris·HCl, pH 7.5, for 3 min, heating an additional 10 min at 60°C, and slowly cooling the mixture to room temperature.

Mutagenesis was performed by incubation of gapped molecules in reaction mixtures containing 3 M sodium bisulfite and 0.5 mM hydroquinone (total volume, 200 μl) at 37°C for 45–90 min (5). Gapped molecules were isolated to remove the mutagen, repaired, and used to transform *Escherichia coli* strain JM101 (6, 7). Single-stranded DNA was purified from isolated plaques and sequenced (adenine and guanine reactions) as described by Sanger *et al.* (8). HSP clones 1–7 and 10 and LSP clones 3–7 and 10–13 were subjected to complete sequence analyses.

***In Vitro* Transcription Analysis.** Closed-circular, double-stranded (viral RF) DNA was purified from individual mutagenized clones from 100-ml cultures of infected cells (JM101) as described by Maniatis *et al.* (9). RF DNAs from clones obtained by mutagenesis of H-strand sequences containing the HSP were digested with *Ava* I; those from L-strand mutagenesis of the HSP were digested with *Eco*RI/*Bgl* II. DNAs from clones obtained by mutagenesis of H- or L-strand sequences containing the LSP were digested with *Eco*RI/*Bgl* II or *Eco*RI, respectively. Digested DNA (0.2–2 μg) was added to *in vitro* transcription reaction mixtures (total volume, 50 μl) containing 5 μl of the heparin-Sepharose fraction of mtRNA polymerase activity {in 10 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/1 mM dithiothreitol/150 μM NTPs (no GTP)/100 mg of bovine serum albumin per ml/0.1 μM [α-^{32}P]GTP} and incubated at 28°C for 30 min (10). Transcription products were fractionated on 7 M urea/6% polyacrylamide gels.

RESULTS

Mutagenesis of the H-Strand Transcriptional Initiation Site. Analyses of the *in vitro* transcription products of an ordered series of deletion clones have demonstrated that accurate H-strand transcription requires only a small region encompassing the initiation site (1, 2). We constructed a series of clones containing point mutations in the sequence proximal to and including the H-strand transcriptional initiation site. Mutagenesis was accomplished by selectively exposing the targeted region, as single-stranded DNA, to sodium bisulfite (Fig. 1) (5, 6). This chemical mutagen efficiently

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Abbreviations: H strand, heavy strand; HSP, heavy-strand promoter; L strand, light strand; LSP, light-strand promoter; nt, nucleotide(s); RF, replicative form.

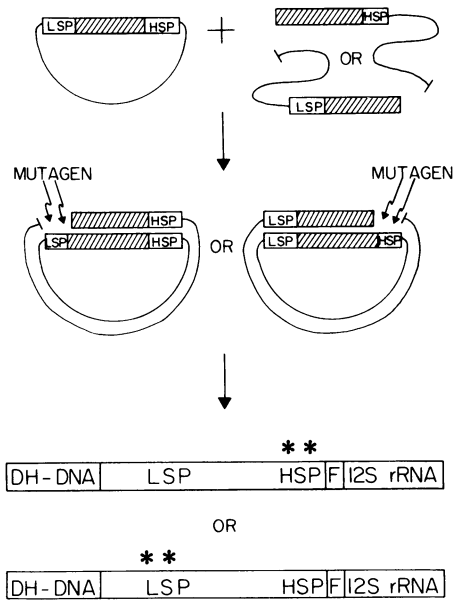

FIG. 1. Site-directed mutagenesis of the HSP and LSP. (*Upper*) Sequences containing the HSP or LSP were exposed as single-stranded regions for sodium bisulfite mutagenesis in separate experiments in which single-stranded DNA from recombinant M13 phage that contains both promoters was mixed with linearized double-stranded DNA that has deleted either the HSP (HSP mutagenesis) or LSP (LSP mutagenesis) (top of figure). (*Lower*) Regions that have been mutagenized are indicated (*) with respect to a genetic map of mtDNA. Each clone contains mutations in only one promoter region, while retaining an unchanged promoter sequence whose activity provides an internal control for *in vitro* transcription assays.

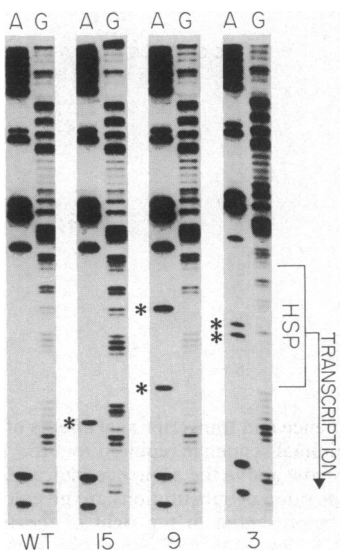

FIG. 2. Adenine (A) and guanine (G) sequence analysis of wild-type and HSP mutagenesis clones. After mutagenesis and transformation, screening of individual clones for mutations was accomplished by sequence analysis using A and G chain-termination sequencing reactions. The occurrence of a C→T substitution in a clone results in the simultaneous appearance of an A residue and loss of a G residue at the site of mutation. The positions of induced mutations in clones 15, 9, and 3 are indicated (*) with respect to the wild-type sequence (WT). The location of the HSP and the site and direction of H-strand transcription, with respect to the sequencing reactions, are indicated to the right of the figure.

causes deamination of non-base-paired cytosine residues in single-stranded DNA, resulting in C→T transitions. Sequences containing C→T or G→A transitions were obtained by mutagenesis of each strand of mtDNA.

Recombinant M13 clones used for mutagenesis were constructed by insertion into M13 mp8 of the mtDNA products of *Eco*RI/*Bam*HI restriction digestion of recombinant pBR322 deletion clones. Gapped molecules were constructed to expose specific regions of each strand of mtDNA by annealing linearized double-stranded DNA (viral RF form) to single-stranded recombinant phage (Fig. 1). For example, C→T transitions were obtained by exposing the L strand from position 511 to position 573 as single-stranded DNA in gapped molecules constructed by annealing the *Bam*HI-linearized RF form of clone H3′Δ−47 to a single-stranded recombinant phage that contains the HSP, H3′Δ+13. Similarly, G→A transitions were obtained by exposing the H strand (positions 498–585) to mutagenesis by annealing the *Bam*HI-linearized RF form of H5′Δ+29 to single-stranded DNA from H5′Δ−60.

Gapped molecules were then treated with sodium bisulfite under conditions that should cause deamination of ≈10% of the cytosine residues in single-stranded DNA (5). The mutagen-treated DNA was purified as described by Folk and Hofstetter (6), and the gap was filled by incubation with the Klenow fragment of *E. coli* DNA polymerase I in the presence of dNTPs. The entire mix was then used to transform the bacterial host strain JM101 (7). Individual plaques were chosen for direct sequence analysis to determine the number and position of C→T substitutions (8). The screening procedure was facilitated by using only adenine and guanine sequencing reactions, since C→T transitions in the phage DNA will result in the loss of a guanine residue and a simultaneous gain of an adenine residue in the sequencing ladder. Fig. 2 shows an example of the determination of sequence substitutions present in several recombinant clones resulting from these mutagenesis experiments. Under the conditions used for mutagenesis, ≈10% of the clones examined were substituted at a single position, 30% at two positions, and 50% at three or more positions. Clones chosen for transcriptional analyses are listed in Fig. 3.

Effects of Mutations on *in Vitro* Transcriptional Activity from the HSP. In order to test the effects of specific base substitutions on transcriptional activity, we analyzed the transcripts of *in vitro* run-off assays of mutagenized clones. Closed-circular, double-stranded DNA was purified from mutagenized and wild-type recombinant M13 clones (viral RF form) and digested with *Eco*RI and *Bgl* II (C→T mutants) or *Ava* I (G→A mutants). Restriction fragments were used as templates for the partially purified mitochondrial RNA polymerase, which faithfully transcribes from the HSP and LSP (1). Transcription that initiates at specific sites within truncated DNA templates produces transcripts of discrete length when the RNA polymerase reaches the end of the template. Run-off transcripts of each clone were resolved by electrophoresis on denaturing 6% polyacrylamide gels.

The recombinant M13 clone used to obtain C→T transitions around the HSP also contains the start site of L-strand transcription, situated within the LSP. The products of run-off transcription of this wild-type DNA are displayed in Fig. 4, lane H. The sizes of the two major products correspond exactly to those expected from accurate initiation at the HSP and LSP. Transcription from the HSP terminates at the site of *Bgl* II cleavage, resulting in the larger of the two run-off products [690 nucleotides (nt)]. Transcription from the LSP terminates at the site of *Eco*RI cleavage, producing the smaller product (407 nt). The presence of an active LSP provides an internal control in assessing the effects of specific alterations on transcription from the HSP. Inhibition of transcriptional activities due to reaction conditions rather than mutations at specific sites would result in loss of activity at the wild-type LSP.

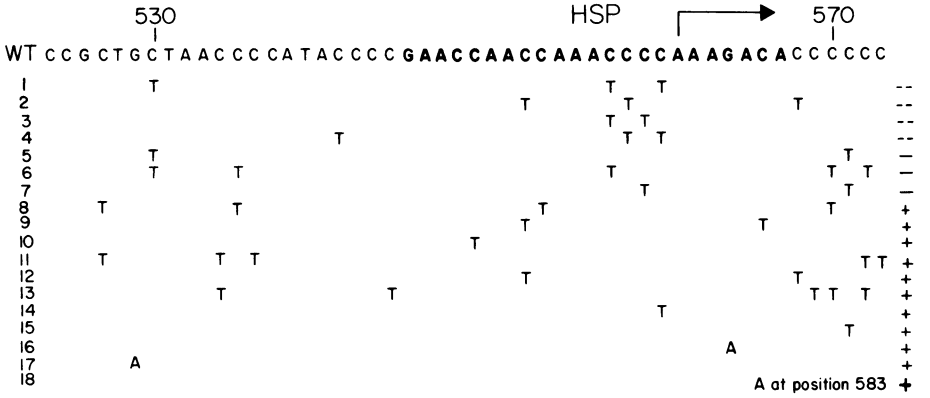

FIG. 3. Sequence and transcriptional effects of HSP mutagenesis clones. The L-strand sequence of wild-type (WT) mtDNA adjacent to and including the minimal sequence required for HSP function (nucleotides in bold lettering) (1) is presented and numbered according to Anderson *et al.* (11). An arrow above the sequence indicates the initiation site and direction of transcription from the HSP. For each mutagenized clone, the nature and position of substitutions are given below the wild-type sequence. The ability of each clone to direct *in vitro* run-off transcription from the HSP is presented to the right of the figure (--, no detectable HSP products; −, barely detectable HSP products at high DNA concentrations; +, levels of HSP expression identical to that of the wild-type clone).

The sequences of a series of clones containing different chemically induced transitions in the region encompassing the start site of H-strand transcription are shown, relative to the wild-type sequence, in Fig. 3. Transcription from the LSP occurred at normal levels in assays of both mutagenized and wild-type clones (Fig. 4), demonstrating that each *in vitro* reaction is equally capable of directing transcription. Differences were seen in the ability of mutagenized clones to direct transcription from the H-strand start site, as indicated by the presence or absence of the larger 690-nt product. The effects on transcriptional activities of G→A transitions in sequences containing the HSP were determined for clones 16, 17, and 18. These mutant clones are capable of directing transcription from the HSP at levels identical to that of the wild-type clone (data not shown). The ability of each mutagenized clone to direct H-strand transcription is presented adjacent to its respective sequence changes in Fig. 3.

Of the 18 mutagenized clones examined, 11 supported normal levels of transcriptional activity from the HSP (Fig. 3). These transcriptionally competent clones contain a varying number of mutated positions (clones 8–18) that flank the start site of H-strand transcription (position 561, Fig. 3). The mutagenized clones that are unable to support *in vitro* transcription from the HSP, with one exception, contain C→T transitions at positions 557–559 (clones 1–7).

At low DNA concentrations, the effect of mutations that altered transcription from the HSP appears to be absolute in that *in vitro* transcription was abolished, not quantitatively reduced (Fig. 4). Using 10-fold higher DNA concentrations, we observed extremely low levels of accurate transcriptional activity from 3 of the 18 mutants examined (clones 5–7). Fig. 5 shows the run-off transcripts of clones 5 (lanes D and I) and 6 (lanes B and G) using different DNA concentrations. For comparison, other mutagenized clones (clone 11, lanes E and F; clone 12, lanes C and H), which exhibit normal transcrip-

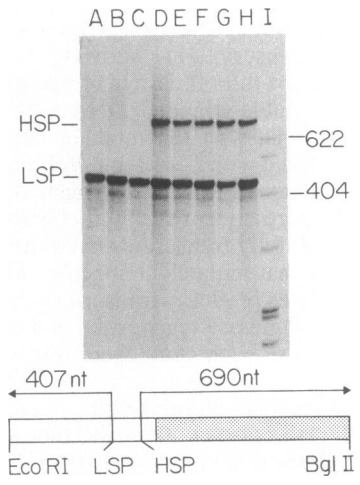

FIG. 4. Products of *in vitro* run-off transcription of HSP mutagenesis clones. A schematic representation of the restriction fragment from each clone used in run-off transcription assays is shown below the autoradiogram. RF DNA from each clone was digested with *Eco*RI and *Bgl* II (the site in the M13 sequence is represented by the stippled region). Specific initiations at the wild-type LSP and HSP produce 407 and 690 nt transcripts, respectively. Above the schematic, an autoradiogram of gel-separated, radiolabeled products of run-off assays with digested templates from various mutagenized clones is presented. Lanes: A, clone 2; B, 3; C, 5; D, 9; E, 14; F, 12; G, 13; H, wild type; I, *Hpa* II-digested pBR322 molecular weight markers. The larger of the two products emanates from the HSP, and the smaller, from the LSP as indicated.

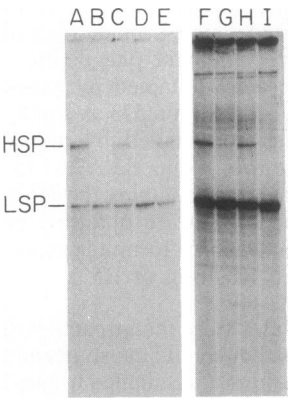

FIG. 5. Products of *in vitro* run-off transcription from HSP mutants at low and high DNA concentration. Lanes A–E show the electrophoretic analysis of radiolabeled products of run-off transcription of several mutagenized clones under assay conditions of low DNA concentration (4 μg/ml): A, wild type; B, clone 6; C, clone 12; D, clone 5; E, clone 11. Lanes F–I show transcripts for the same mutagenized clones under assay conditions of 10-fold higher DNA concentration (40 μg/ml): F, clone 11; G, clone 6; H, clone 12; I, clone 5. Examination of lanes G and I (clones 5 and 6) reveals small, but detectable, amounts of transcription at high DNA concentrations from the HSP, although at low DNA concentrations (lanes B and D), loss of transcription from the HSP appears to be absolute.

tional activities, are included. It is clear from close examination of these transcription products that clones 5 and 6 directed correct transcription from the HSP at low levels under assay conditions of high DNA concentration. Mutations at positions that did not affect H-strand transcription directed normal levels of transcriptional activity. In this class of templates, there was no correlation between the number of mutagenized positions and the level of transcription. In the analysis of mutagenized clones, we did not observe H-strand transcription from other sites or increased levels of nonspecific initiation when normal activity was altered.

Mutagenesis of the L-Strand Transcriptional Initiation Site. The minimal region of mtDNA required for *in vitro* transcription from the LSP, as determined by analysis of a series of deletion clones, includes the site of initiation (1). In order to define the critical sequences that comprise the LSP, site-directed mutagenesis was used to induce substitutions at specific positions within a region that included the start site of L-strand transcription. By exposing each strand of mtDNA to chemical mutagenesis, altered sequences containing C→T and G→A transitions were obtained. Gapped molecules were constructed to expose the H-strand sequences containing the LSP to mutagenesis by annealing the *Bam*HI-linearized RF form of recombinant M13 clone L3′Δ−58 with the single-stranded DNA from L3′Δ+28. Similarly, clones L5′Δ+1 and L5′Δ−70 were annealed to selectively mutagenize L-strand sequences containing the LSP. Gapped molecules were treated with sodium bisulfite and used for transformation as described above for HSP mutagenesis (Fig. 1). Fig. 6 presents the sequence substitutions in each of the mutagenized mtDNA clones.

Effects of Mutations on *in Vitro* Transcriptional Activity from the LSP. RF DNAs from mutagenized recombinant M13 clones were digested with restriction enzymes and used as templates in run-off transcription assays to determine the effects of base substitutions at various positions on *in vitro* activity from the LSP. Fig. 7 presents an autoradiogram of gel-separated products of *in vitro* transcription assays using templates from clones containing C→T transitions (Fig. 6). These clones contain wild-type HSP sequences whose expression provides an internal control in each reaction. The products of *in vitro* transcription from the HSP and LSP of *Eco*RI/*Bgl* II-digested RF DNA from the wild-type clone were 190 nt and 690 nt, respectively (Fig. 7, lanes A and C). While each mutagenized clone directed transcription from the HSP, the level of transcription from the LSP ranged from no expression (clone 7, lane B) to normal levels of expression (clones 1 and 2, lanes G and H). In order to examine more closely the effects of mutations on transcription from the LSP, a series of *in vitro* assays was performed in which a wide range of DNA concentrations was tested for each mutagenized clone (data not shown). These experiments showed that

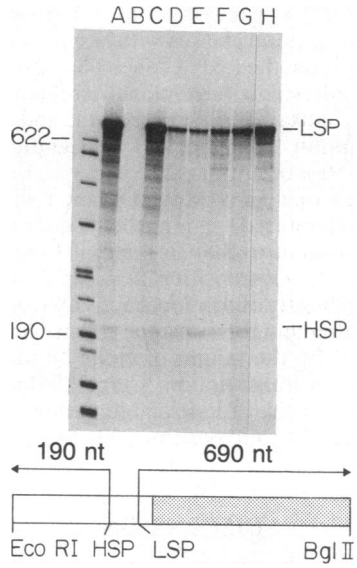

FIG. 7. *In vitro* run-off transcription products of LSP mutagenesis clones. At the bottom of the figure, a schematic representation of the restriction fragment used in run-off assays of LSP mutagenesis clones is presented. Specific initiations at the wild-type HSP and LSP produce 190 and 690 nt transcripts, respectively. Above the schematic, an autoradiogram of the gel-analyzed, radiolabeled products of *in vitro* transcription of wild-type and various LSP mutagenesis clones is shown. Lanes: A, wild type; B, clone 7; C, wild type; D, clone 6; E, clone 5; F, clone 4; G, clone 1; H, clone 2. The smaller of the two major products is from the HSP, and the larger, from the LSP. Molecular weight markers are based on the radiolabeled *Hpa* II digests of pBR322 DNA.

reductions of LSP transcriptional activity on mutagenized clones do not result from minor differences in DNA concentration but reflect impaired transcriptional efficiencies over a wide range of DNA concentrations.

Mutagenized clones containing G→A transitions (Fig. 6) were assayed to determine the effects of specific base substitutions at defined positions on transcription from the LSP. RF DNA from these clones was digested with *Eco*RI and used as the template in run-off transcription assays. Because these clones do not contain the HSP, restriction fragments containing nonmutagenized LSP sequences, which produce transcripts of a different size, were added as an internal control for each reaction. All of the mutagenized clones containing G→A transitions were capable of directing transcription from the LSP, although several (clones 10–13) showed a reduction in levels of transcription.

The positions of sequence substitutions in a series of mutagenized clones and their effects on *in vitro* transcription

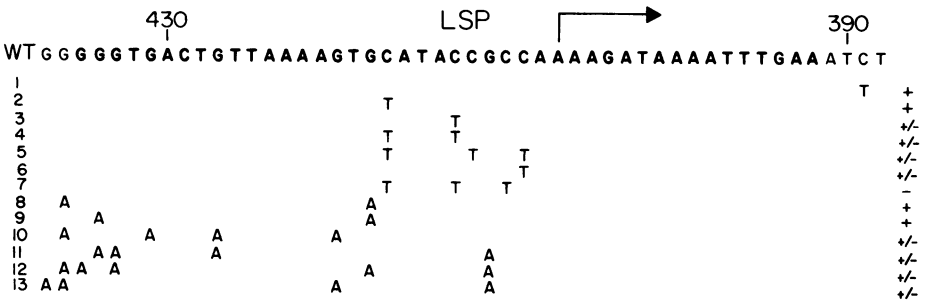

FIG. 6. Sequence and transcriptional effects of LSP mutagenesis clones. The H-strand sequence of wild-type mtDNA, including the start site of L-strand transcription (arrow above sequence) and the minimal region required for transcription of the LSP (nucleotides in bold lettering) (1), is given and numbered according to Anderson *et al.* (11). For each clone, the position of induced mutations is shown below the wild-type sequence. The ability of each clone to direct transcription from the LSP is given to the right of the figure (+, activity identical to that of the wild-type clone; +/−, 50–80% reduction of wild-type activity; −, no detectable activity from the LSP).

from the LSP are summarized in Fig. 6. Individual clones fall into one of three general classes with respect to the efficiency of transcription from the LSP. Clones 1 and 2 direct transcription at normal efficiencies (+), clone 7 contains substitutions that drastically curtail LSP activity (−), and the remainder direct transcription from the LSP at significantly reduced efficiency (20–50% of normal activity; +/−). All but one of the nine clones that showed significant reduction of LSP activity are substituted within the sequence C-C-G-C-C immediately adjacent to the start site of L-strand transcription (Fig. 6). A C→T transition at position 410 within this sequence abolishes transcriptional activity from the LSP. An additional requirement for sequences upstream from the start site is indicated by the failure of clone 10 to direct normal levels of L-strand transcription. Clone 10 does not contain substitutions at the site of L-strand initiation of transcription but is substituted at four positions 13–29 nt upstream from the start site.

DISCUSSION

Critical Regions for Promoter Function. The results of analyses with mutagenized, cloned mtDNAs indicate that the region required for accurate initiation of *in vitro* H-strand transcription is extremely small and includes the nucleotides where initiation occurs (Figs. 3 and 8, positions 557–559). Substitution at these three nucleotides drastically reduces transcription. In addition, substitution at one position (530) located 31 nt upstream from the initiation site (Fig. 3, clone 5) reduces H-strand transcription, providing additional evidence for the existence of flanking control regions (1). From this analysis, it appears that this putative upstream control region is very small, as multiple substitutions at positions adjacent to position 530 do not affect H-strand transcription. We further note the positive transcription on clone 16 (Fig. 3), which is identical in sequence to a conserved sequence (CSB-3) that occurs downstream of LSP in the displacement-loop region (10) and which does not support transcriptional initiation in its natural location (1). Any potential role for flanking sequences in modulating mitochondrial promoter function is not yet known.

It is clear that the LSP is less sensitive to sequence alteration (Figs. 6 and 8). A change at only one position, three nucleotides upstream of the transcriptional start site, causes complete abolition of L-strand transcription. However, the requirement for immediately upstream cytosine residues is a general one for both the HSP and LSP.

A Minimal Mitochondrial Promoter. One can infer that a very short sequence comprised of 3′ C-C-C-C-A-A-A-(A) 5′ may be capable of supporting at least a minimal amount of transcriptional initiation near the boundary position between cytosines and adenines. This view is consistent with recent promoter mapping studies (1, 2). The mutagenesis data show that the cytosines are critical to expression; a presumed necessity for the adenines remains to be determined.

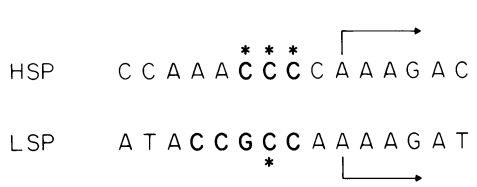

FIG. 8. Identification of nucleotides important in HSP and LSP function. mtDNA sequences from the HSP and LSP are aligned with respect to the start sites of H- and L-strand transcription (1). Nucleotides in bold lettering represent positions where induced mutations cause a 50–80% reduction in promoter activity. Nucleotides in bold lettering with asterisks indicate positions that are absolutely required for promoter function. Induced mutations at these sites effectively abolish promoter activity.

Because cloned, linear restriction fragments support transcription in this system with the same degree of initiation precision as do cloned, closed-circular plasmids or closed-circular mtDNA, the following conclusions are warranted. Neither a closed-circular topology nor the displacement-loop structure itself is required for accurate promoter function. The degree of methylation normally associated with DNAs propagated in standard laboratory *E. coli* strains is not inhibitory nor is it plausible that some form of base modification inherent to eukaryotic mitochondria is operative. We note, however, that some or all of these features could affect the efficiency of promoter function; this remains to be tested.

Are Mammalian Mitochondrial Promoters Conserved Elements? The functional demands on the LSP differ markedly from those of the HSP. The HSP has thus far been implicated only in the role of a transcriptional promoter for generation of structural RNAs. In contrast, the LSP is also the site of priming of H-strand replication (12) and therefore both transcription of L-strand genes and mtDNA replication are served by this promoter. In addition, it is known that human mitochondria contain a short RNA species whose 5′ end maps at this promoter (4), and it is possible that this reflects a separate synthetic event. For these reasons it seems possible that a more complex set of proteins interacts with the LSP compared to the HSP. If so, both the primary sequence and sensitivity to mutagenesis differences between the HSP and LSP may reflect this situation.

Despite the apparent simplicity of human mitochondrial promoters, the fact remains that such regulatory elements must be species-specific because they are not highly conserved between species for which displacement-loop nucleotide sequence information is available (1). This situation is in distinct contrast to the mammalian origin of L-strand DNA replication, which is highly conserved (3). One could reasonably argue that the organelle DNA polymerase and L-strand-origin priming activity may not differ significantly between species, but that the nuclear-encoded proteins that interact with the HSP and LSP are uniquely tailored for each organism and, therefore, might represent a species barrier at the level of mitochondrial biogenesis.

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