MOLECULAR AND CELLULAR BIOLOGY, Mar. 1989, p. 1200–1211
0270-7306/89/031200-12$02.00/0

Vol. 9, No. 3

Identification of Transcriptional Regulatory Elements in Human Mitochondrial DNA by Linker Substitution Analysis

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Received 26 July 1988/Accepted 30 November 1988

Human mitochondrial DNA contains two major promoters, one for transcription of each strand of the helix. Previous mapping and mutagenesis data have localized these regulatory elements and have suggested regions important to their function. In order to define, at high resolution, the sequences critical for accurate and efficient transcriptional initiation, a linker substitution analysis of the entire promoter region was performed. Each promoter was shown to consist of approximately 50 base pairs comprising two functionally distinct elements. These and previous data strongly support a mode of transcription initiation requiring minimal sequences surrounding the initiation sites that are likely interactive with core polymerase and upstream regulatory domains capable of binding a transcription factor that modulates the efficiency of transcription initiation. Furthermore, in at least one case, this upstream regulatory domain is capable of operating bidirectionally.

At the onset of this study, our understanding of the anatomy of the transcriptional control regions of vertebrate mitochondrial DNA (mtDNA) derived from two lines of data. First was the work of Walberg and Clayton (37), who established the ability of a mitochondrial extract to transcribe cloned mtDNA in vitro accurately. The development of an in vitro assay for correctly initiated transcription permitted Chang and Clayton (12) to characterize the regions of the displacement loop (D loop) responsible for supporting accurate initiation by a systematic deletion-mutagenesis approach; this resulted in the definition of two major promoters, one for the light (L) strand and one for the heavy (H) strand. An analysis of a number of point mutants by Hixson and Clayton (25) extended these observations. A second body of information consisted of a biochemical analysis of the transcriptional machinery, which revealed a separable transcription factor that promoted accurate and efficient transcription in the presence of a largely nonselective RNA polymerase (20). This transcription factor binds to DNA upstream of the transcriptional start sites of both the L-strand promoter (LSP) and H-strand promoter (HSP) (22). Both lines of evidence were consistent with the concept that a relatively small region surrounding the start site was necessary and sufficient for accurate transcriptional initiation, whereas the upstream sequences influenced transcriptional efficiency.

To define systematically the sequences responsible for both accurate and efficient transcription, a linker substitution mutagenesis approach (24, 31) was used to generate small clusters of multiple point mutations throughout the entire region surrounding the two promoters. Subsequent analyses of these mutants, in the presence of both promoters and without the introduction of foreign vector sequences, delineated the upstream efficiency domains of human mitochondrial promoters and refined the boundary between this upstream domain and the corresponding downstream sequences surrounding the start site that are required for transcriptional initiation.

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MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases and DNA-modifying enzymes were purchased from the following commercial suppliers: Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), New England BioLabs, Inc. (Beverly, Mass.), and P-L Biochemicals, Inc. (Milwaukee, Wis.). Nucleoside triphosphates and poly(dA-dT) were obtained from P-L Biochemicals. [α-^{32}P]GTP and [α-^{32}P]dATP were obtained from Amersham Corp. (Arlington Heights, Ill.). *Bam*HI linkers were purchased from Collaborative Research, Inc. (Waltham, Mass.).

Construction of mutants. All mutants were derived from two parental clones containing nucleotides (nt) 1 to 622 and 360 to 741 of human KB cell mtDNA, inserted into the *Bam*HI and *Eco*RI sites of M13mp8 (provided by D. D. Chang of this laboratory). Deletions were constructed by linearizing the replicative forms of these clones at the *Bam*HI sites situated at nt 622 or 360, respectively. Linearized DNA was preincubated in 20 mM Tris hydrochloride (pH 8.0)–12 mM $CaCl_2$–50 μg of bovine serum albumin per ml at 30°C for 10 min. From 0.5 to 2.5 U of BAL 31 was added, and equal portions were removed at 30-s intervals; the reaction was terminated by the addition of EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid] to 20 mM. The extent of deletion was assayed by agarose gel electrophoresis. Equal portions with the appropriate extents of deletion were pooled, purified by phenol-chloroform extraction, and precipitated with ethanol. The staggered ends left by BAL 31 were repaired with T4 DNA polymerase in the presence of 1 mM deoxynucleoside triphosphates. *Bam*HI linkers, 5′-GGCCTAGGCC-3′, were added, and the deleted inserts were excised with *Eco*RI. The resulting fragments were gel isolated and subcloned directly into *Bam*HI-*Eco*RI-cleaved M13mp8RF; deletion endpoints were mapped directly by dideoxy sequencing. Individual linker substitution (LS) mutants were constructed by joining two nonoverlapping deletion clones, whose ends differed by 10 ± 2 base pairs (bp), at their *Bam*HI sites. In the process of joining the respective deletion clones, the mutants were subcloned into pBR322 along with some flanking M13mp8 sequences; it was in this form that all

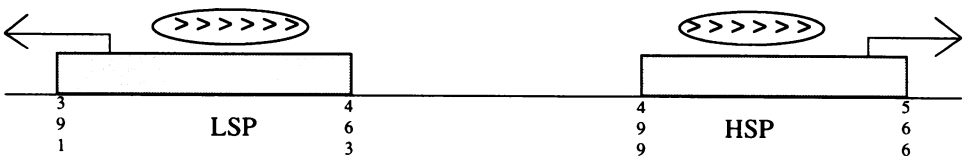


FIG. 1. Schematic of human mitochondrial promoters. The LSP and HSP of human mtDNA are shown in their relative positions at the D-loop region. The major start sites at nt 407 and 559 as well as the directions of transcription are indicated by arrows. Boxes encompass the nucleotides required for a wild-type level of transcription in vitro as defined by the initial deletion analysis of Chang and Clayton (12). Nucleotide numbers here and throughout are those of human KB cell mtDNA (12). The approximate site of binding by the mitochondrial transcription factor mtTF1 is shown; arrowheads within ovals represent the orientation with which this factor binds to each promoter (see text).

subsequent analyses took place. In the case of the hybrid promoter mutants (HPM), the procedure was identical except that the deletions differed in their endpoints by substantially more than 10 bp, and therefore their recombination resulted in a deletion of mitochondrial sequences. All LS clones and HPM mutants were linearized with *Eco*RI for transcriptional analysis. LSP deletions, pKB410(Δ411-741) and pKB419(Δ420-741), were assayed for ability to support transcription by linearization at the single *Eco*RI site either directly in the M13RF form or after subcloning into pBR322. The LS and HPM mutants are designated by the numbers of the nucleotides (12) deleted and replaced by the linker. For example, LS 411/420 has had nt 411 through 420, inclusive, deleted and a *Bam*HI linker inserted in their place. Similarly, HPM 411/517 has had nt 411 through 517, inclusive, deleted and replaced by a *Bam*HI linker.

An extract of mitochondria containing both mtRNA polymerase and mitochondrial transcription factor 1 (mtTF1) was prepared from human KB cells by a modification of the procedures of Walberg and Clayton (37) and Fisher and Clayton (20). Briefly, mitochondria were isolated as described by these authors and lysed in the presence of 10% glycerol, 20 mM Tris hydrochloride (pH 8.0), 0.2 M KCl, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride and dithiothreitol by vortexing every 5 min for 15 min. The lysate was cleared by centrifugation at 100,000 × *g* for 1 h, diluted twofold, and loaded onto a DEAE-Sephacel column equilibrated with buffer A–0.1 M KCl (buffer A is 20 mM Tris hydrochloride [pH 8.0], 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol). The column was washed with 10 column volumes of buffer A–0.1 M KCl and eluted with buffer A–0.3 M KCl. Fractions were assayed for mtRNA polymerase activity on a poly(dA-dT) template as described previously (20). Active fractions were pooled and dialyzed into 50% glycerol plus buffer A–50 mM KCl.

Transcription reactions were carried out in 25-μl volumes containing 20 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 50 μg of bovine serum albumin per ml, 1 mM dithiothreitol, 400 μM ATP, UTP, and CTP, 4 μM GTP, and 6 μCi of [^{32}P]GTP (800 Ci/mmol). Template concentration was varied from 2 to 4 μg/ml, and the typical transcription reaction contained 2 μl of the pooled DEAE fraction (~2 μg of protein). The reaction was incubated for 20 min at 28°C and terminated by the addition of 75 μl of stop buffer containing 133 μg of proteinase K per ml–2% sodium dodecyl sulfate–20 mM EDTA–20 mM Tris hydrochloride (pH 7.35) and incubated for an additional 10 min at 37°C. After two phenol-chloroform extractions, the RNA was precipitated with ethanol in the presence of ammonium acetate and analyzed on a 6% polyacrylamide–7 M urea gel as described elsewhere (37).

RESULTS

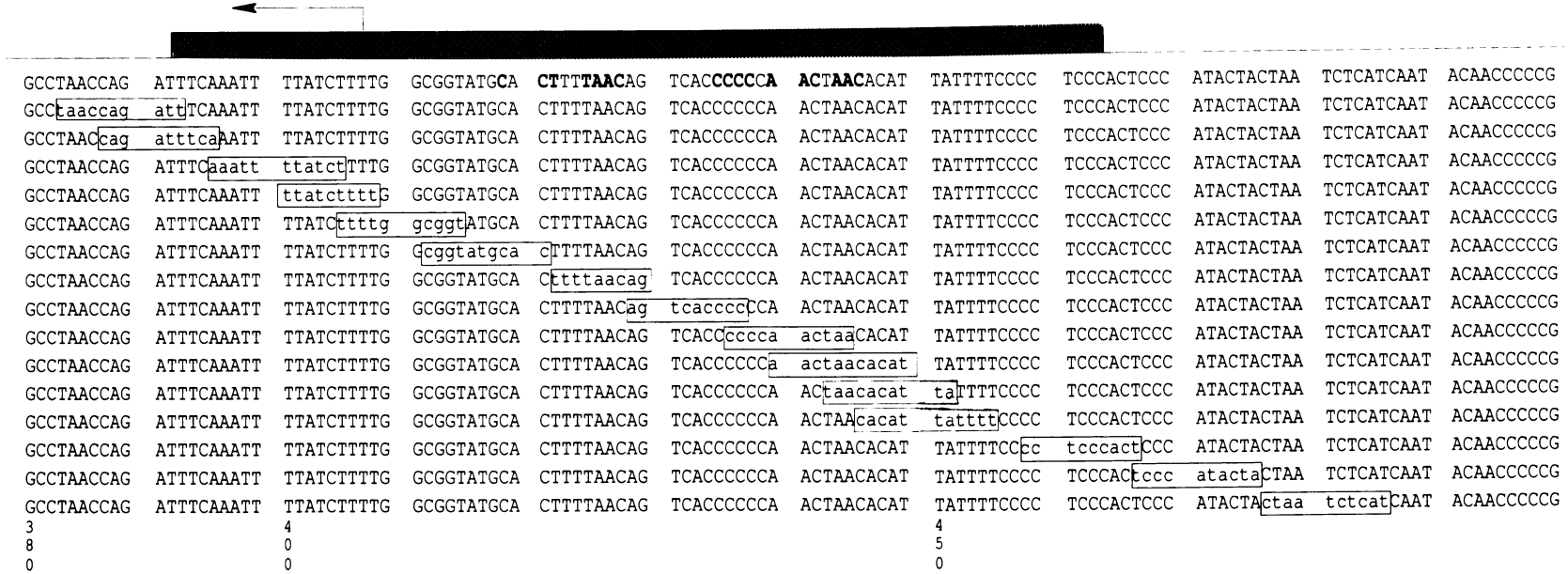
Linker substitution analysis. By using the procedures outlined in Materials and Methods, a total of 30 LS mutants were constructed. The mutants spanned essentially the entire transcriptional control region surrounding the two transcriptional start sites at the LSP and HSP, situated at nt 407 and 559, respectively (12).

Figure 1 depicts the overall organization of the two promoters of human mtDNA. These major start sites are denoted by arrows, and the boxed region represents the nucleotides responsible for maximal transcription in vitro as defined by the initial deletion analysis of Chang and Clayton (12). mtTF1 (20–22) is shown binding to the template upstream of both start sites. The regions upstream of both promoters that interact with mtTF1 contain a limited sequence homology that is present in opposite orientations with respect to the major start sites of transcription of the LSP and HSP, respectively (22). Thus, the transcription factor presumably binds in opposite orientations within the two promoters (Fig. 1, arrowheads within ovals).

The LS mutants have been separated into LSP LS mutants 383/392 through 476/485 and HSP LS mutants 501/511 through 601/608 for the purposes of discussion only. It is important to point out that both promoters are present on the same DNA fragment for nearly all of these analyses, thereby providing a positive control of a wild-type promoter within the same template as the mutant promoter.

Scanning of the LSP. The sequences of the LS mutants surrounding the LSP are shown in Fig. 2A. The boxed nucleotides are those that have been deleted and replaced with the 10-bp *Bam*HI linker. The top line of Fig. 2A is a schematic of the LSP. As in Fig. 1, the major start site of transcription is indicated as an arrow and the boxed region represents the region sufficient for a wild-type level of transcription (12). Figure 2B is an autoradiogram of a typical in vitro transcription experiment, using nine representative LSP-LS mutants as templates. The prominent runoff transcript of 410 nt resulted from accurate initiation at the LSP located at nt 407 ± 1. The prominent species at 180 nt was a runoff transcript resulting from accurate initiation at the HSP present on the same DNA fragment. As seen in a wild-type transcription reaction, several other bands in the ranges of 300 to 400 nt and 90 to 100 nt were commonly seen. These species were all LSP dependent and likely resulted from either processing or premature termination of the transcript occurring during the transcription reaction. In most cases, the amounts of these shorter transcripts were proportional to the amount of full-length transcript. Table 1 displays the relative transcriptional activities of all 15 LSP LS mutations.

A


WT GCCTAACCAG ATTTCAAATT TTATCTTTTG GCGGTATGCA CTTTTAACAG TCACCCCCCA ACTAACACAT TATTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 383/392 GCCtaaccag attTCAAATT TTATCTTTTG GCGGTATGCA CTTTTAACAG TCACCCCCCA ACTAACACAT TATTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 387/395 GCCTAACcag atttcaAATT TTATCTTTTG GCGGTATGCA CTTTTAACAG TCACCCCCCA ACTAACACAT TATTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 395/405 GCCTAACCAG ATTTCaaatt ttatctTTTG GCGGTATGCA CTTTTAACAG TCACCCCCCA ACTAACACAT TATTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 400/408 GCCTAACCAG ATTTCAAATT ttatcttttG GCGGTATGCA CTTTTAACAG TCACCCCCCA ACTAACACAT TATTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 405/414 GCCTAACCAG ATTTCAAATT TTATCtttg gcggtATGCA CTTTTAACAG TCACCCCCCA ACTAACACAT TATTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 411/420 GCCTAACCAG ATTTCAAATT TTATCTTTTG Gcggtatgca cTTTTAACAG TCACCCCCCA ACTAACACAT TATTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 421/429 GCCTAACCAG ATTTCAAATT TTATCTTTTG GCGGTATGCA Ctttaacag TCACCCCCCA ACTAACACAT TATTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 428/436 GCCTAACCAG ATTTCAAATT TTATCTTTTG GCGGTATGCA CTTTTAACag tcaccccCCA ACTAACACAT TATTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 435/444 GCCTAACCAG ATTTCAAATT TTATCTTTTG GCGGTATGCA CTTTTAACAG TCACCcccca actaaCACAT TATTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 439/449 GCCTAACCAG ATTTCAAATT TTATCTTTTG GCGGTATGCA CTTTTAACAG TCACCCCCCa actaacacat TATTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 442/451 GCCTAACCAG ATTTCAAATT TTATCTTTTG GCGGTATGCA CTTTTAACAG TCACCCCCCA ACtaacacat taTTTTCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 445/455 GCCTAACCAG ATTTCAAATT TTATCTTTTG GCGGTATGCA CTTTTAACAG TCACCCCCCA ACTAAcacat tattttCCCC TCCCACTCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 458/466 GCCTAACCAG ATTTCAAATT TTATCTTTTG GCGGTATGCA CTTTTAACAG TCACCCCCCA ACTAACACAT TATTTTCCcc tcccactCCC ATACTACTAA TCTCATCAAT ACAACCCCCG
LS 466/475 GCCTAACCAG ATTTCAAATT TTATCTTTTG GCGGTATGCA CTTTTAACAG TCACCCCCCA ACTAACACAT TATTTTCCCC TCCCACtccc atactaCTAA TCTCATCAAT ACAACCCCCG
LS 476/485 GCCTAACCAG ATTTCAAATT TTATCTTTTG GCGGTATGCA CTTTTAACAG TCACCCCCCA ACTAACACAT TATTTTCCCC TCCCACTCCC ATACTActaa tctcatCAAT ACAACCCCCG
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0 0 0

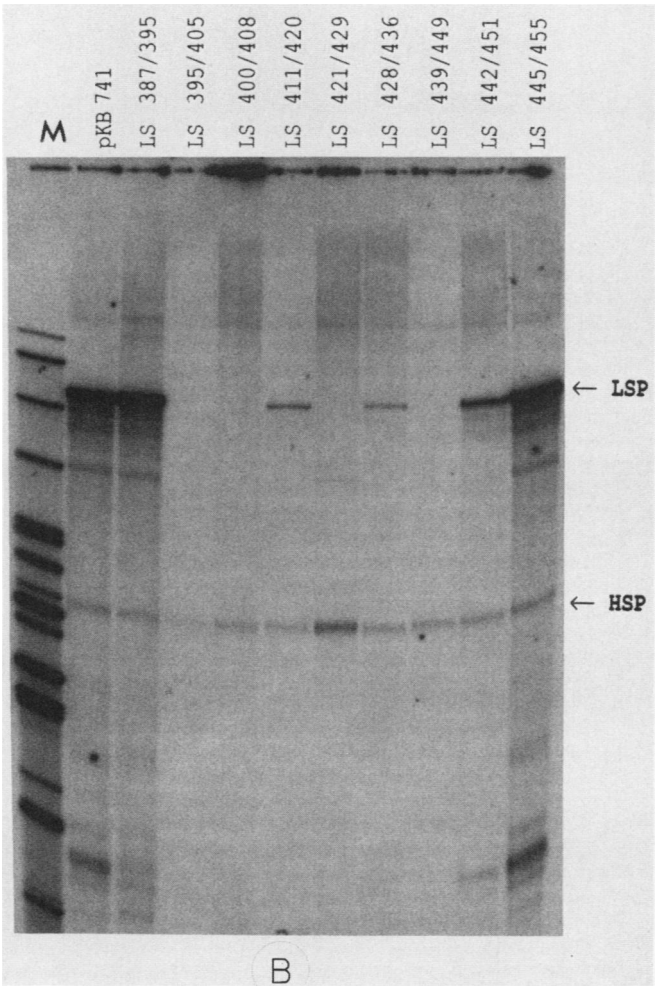

FIG. 2. Sequences and transcriptional phenotypes of LS mutants scanning the LSP. (A) The top line contains the wild-type sequence from nt 380 to 499. This strand represents the coding strand for transcription emanating from the LSP. The same strand of DNA is displayed in Fig. 3, where it represents the HSP noncoding strand. The boldface nucleotides indicate the conserved bases present in the upstream regions of both the LSP and the HSP (22). The schematic above the wild-type (wt) sequence indicates the start site of LSP transcription as well as the maximal extent of sequences required for wild-type transcription in vitro (defined by the initial deletion analysis as displayed in Fig. 1). The 15 LS mutants scanning the LSP are shown below the wild-type sequence. Numerical designations refer to the nucleotides in lowercase that are boxed; in each mutant, it is these nucleotides that have been deleted and replaced by a *Bam*HI decamer 5′-CCGGATCCGG-3′. Five of the LSP LS mutants presented here, LS 405/414, LS 411/420, LS 428/436, LS 435/444, and LS 439/449, have appeared previously; the previous nomenclature denoted them as LS+3/−7, LS−4/−13, LS−21/−29, LS−28/−37, and LS−32/−42, respectively (22). (B) Autoradiogram of a typical in vitro transcription experiment. M, *Hpa*II-cleaved pBR322 DNA used as size marker. pKB741 is the wild-type transcription template containing both promoters. The nine LS mutants assayed are indicated; positions of the expected runoff transcripts from both the LSP and HSP are indicated at approximately 410 and 180 nt, respectively.

The most striking feature of the LSP analysis was the observation of multiple, separable control regions. The first consisted of the transcriptional start site itself and a small amount of flanking sequence. Mutants as close as 12 bp

TABLE 1. Transcriptional competence of LS mutants surrounding the LSP

Clone	Level of transcription[a]	
	LSP	HSP
LS 383/392	100	100
LS 387/395	100	100
LS 395/405	0	95
LS 400/408	0	101
LS 405/414	0	95
LS 411/420	12	100
LS 421/429	1	156
LS 428/436	10	100
LS 435/444	2	111
LS 439/449	1	119
LS 442/451	38	97
LS 445/455	103	100
LS 458/466	100	100
LS 466/475	102	94
LS 476/485	98	99

[a] The transcriptional phenotypes are expressed as percentage of wild-type activity as defined by transcription from pKB741. Quantification was by densitometry performed on the original autoradiograms, and relative levels of transcription were calculated from wild-type controls performed with each experiment. Values are averages of at least three independent experiments.

downstream of the LSP start site, such as 387/395, had no observable effect on the level of LSP runoff transcripts. However, three mutants, 395/405, 400/408, and 405/414, all of which altered the template initiation site itself or bases very close to it, reduced LSP transcription to undetectable levels. All of these start site mutations consisted of multiple base changes within the minimal promoter and the LSP-HSP start site consensus proposed by Chang and Clayton (12). Interestingly, mutant 395/405 altered only the 3′-most A of the proposed 5′-CCCAAA(A)-3′ minimal mitochondrial promoter of Hixson and Clayton (25), which implied that this nucleotide was essential for any initiation to occur or that a few more nucleotides 3′ of this position were also required. LS mutant 411/420, which did allow significant, though reduced, levels of initiation, placed the upstream border of sequences required for accurate initiation at approximately nt 411. As noted by Fisher et al. (22), this mutant may have been situated such that it defined a transition point between start site sequences and sequences involved in upstream recognition by mtTF1. However, care must be taken when evaluating the effect of this mutant for the following reason. When the 5′ deletion ending at nt 410 [pKB 410(Δ411-741), which also had the *Bam*HI linker and thus was identical to LS mutant 411/420 up to −13 of the LSP start site but continued into vector sequences instead of the upstream mitochondrial sequences present in the LS mutant] was assayed in the in vitro system, no detectable transcription was observed. A deletion to nt 419 [pKB419(Δ420-740)] also did not support any detectable transcription in the absence of upstream mitochondrial sequences (see Fig. 4B). This finding implied that sequences upstream of nt 419 were required for any accurate initiation to occur at the LSP at the level of detection afforded by this system.

As the mutations moved upstream of the start site, they defined a region from approximately nt 412 to 446 that influenced the efficiency of initiation while sparing the ability of the RNA polymerase to initiate accurately. These mutants displayed a range of transcriptional deficits; mutants such as LS 421/429, 435/444, and 439/449 had less than 5% of the wild-type level of LSP activity, mutants LS 411/420 and 428/436 had approximately 10% of wild-type activity, and

A

		5′										3′
WT		CCCATCCTAC	CCAGCACACA	CACCGCTGCT	AACCCCATAC	CCCGAACCAA	CCAAACCCCA	AAGACACCCC	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	501/511	C-----------	--AGCACACA	CACCGCTGCT	AACCCCATAC	CCCGAACCAA	CCAAACCCCA	AAGACACCCC	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	506/514	CCCATC------	------ACACA	CACCGCTGCT	AACCCCATAC	CCCGAACCAA	CCAAACCCCA	AAGACACCCC	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	510/517	CCCATCCTAC	----------CA	CACCGCTGCT	AACCCCATAC	CCCGAACCAA	CCAAACCCCA	AAGACACCCC	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	517/524	CCCATCCTAC	CCAGCAC-----	----------	AACCCCATAC	CCCGAACCAA	CCAAACCCCA	AAGACACCCC	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	521/529	CCCATCCTAC	CCAGCACACA	----------	AACCCCATAC	CCCGAACCAA	CCAAACCCCA	AAGACACCCC	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	526/538	CCCATCCTAC	CCAGCACACA	CACCGC------	----------	CCCGAACCAA	CCAAACCCCA	AAGACACCCC	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	536/547	CCCATCCTAC	CCAGCACACA	CACCGCTGCT	AACCCC------	----------	CCAAACCCCA	AAGACACCCC	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	543/551	CCCATCCTAC	CCAGCACACA	CACCGCTGCT	AACCCCATAC	CCC---------	----------	AAGACACCCC	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	552/561	CCCATCCTAC	CCAGCACACA	CACCGCTGCT	AACCCCATAC	CCCGAACCAA	CC----------	----------	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	558/566	CCCATCCTAC	CCAGCACACA	CACCGCTGCT	AACCCCATAC	CCCGAACCAA	CCAAACCC----	----------	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	565/573	CCCATCCTAC	CCAGCACACA	CACCGCTGCT	AACCCCATAC	CCCGAACCAA	CCAAACCCCA	AAGAC------	----------	TGTAGCTTAC	CTCCTCAAAG	CAATACACTG
LS	572/581	CCCATCCTAC	CCAGCACACA	CACCGCTGCT	AACCCCATAC	CCCGAACCAA	CCAAACCCCA	AAGACACCCC	CC----------	----------	CTCCTCAAAG	CAATACACTG
LS	578/589	CCCATCCTAC	CCAGCACACA	CACCGCTGCT	AACCCCATAC	CCCGAACCAA	CCAAACCCCA	AAGACACCCC	CCACAGTTTa	----------	CTCCTCAAAG	CAATACACTG
LS	588/597	CCCATCCTAC	CCAGCACACA	CACCGCTGCT	AACCCCATAC	CCCGAACCAA	CCAAACCCCA	AAGACACCCC	CCACAGTTTA	TGTAGCTTac	----------	CAATACACTG
LS	601/608	CCCATCCTAC	CCAGCACACA	CACCGCTGCT	AACCCCATAC	CCCGAACCAA	CCAAACCCCA	AAGACACCCC	CCACAGTTTA	TGTAGCTTAC	CTCCTCAAAG	C----------

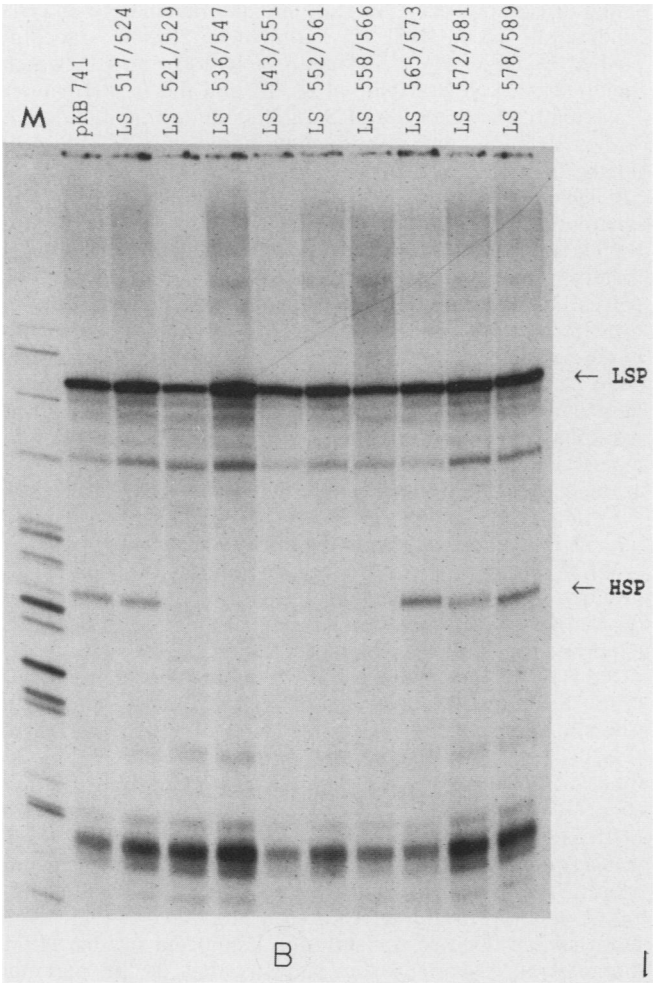

FIG. 3. Sequences and transcriptional phenotypes of LS mutants scanning the HSP. (A) The wild-type (wt) sequence from nt 500 to 609 is shown. The schematic above the sequences indicates the start site of HSP transcription at nt 559, and the maximal sequences required for HSP transcription (defined by deletion analysis [12]) are denoted by the shaded box. The mutants are designated by the nucleotides that have been deleted and replaced by the *Bam*HI linker, as in Fig. 2. These nucleotides are boxed for each mutant. (B) Autoradiogram of a typical in vitro transcription experiment of nine HSP LS mutants. M, *Hpa*II-cleaved pBR322; pKB741, wild-type transcription template. The mutants assayed are shown, and positions of the LSP and HSP runoff transcripts are indicated as in Fig. 2.

mutant LS 442/451 had 38% of wild-type activity. These mutants exhibited only a quantitative effect on the level of transcription, whereas the accuracy of initiation was intact.

The LSP LS mutants that were analyzed previously (22) were also assayed for ability to bind stably to purified mtTF1 in a footprinting assay. Two of these mutants, 435/444 and 439/449, were found to be completely deficient in ability to be footprinted, whereas mutants 411/420 and 428/436 were diminished in ability to be footprinted (22). It was concluded that those results were in good agreement with the transcriptional data and also supported the functional significance of the LSP-HSP upstream homology previously reported (22).

Results of the more complete mutagenesis presented here agreed with previous data and provided additional support for the importance of upstream regions in the control of LSP transcription. There was an excellent correlation between the degree of transcriptional deficit in vitro and the extent to which bases conserved between the LSP and HSP upstream control regions were altered in each LS mutant. Mutants 421/429, 435/444, and 439/449, all three of which were clustered around one of the two conserved domains of the homology, showed the greatest transcriptional deficits. Mutants 411/420, 428/436, and 442/451 fell either at the edges of or between the conserved regions, thus causing fewer changes away from the consensus; as predicted, their transcriptional phenotype was not impaired to the same extent. Furthermore, the approximately 10-bp periodicity displayed by the mutants of the two classes (i.e., 411/420, 428/436, 442/451, which allowed appreciable transcriptional starts, or 421/429 and 435/444, which allowed only a negligible level of transcription) may reflect a similar periodicity in the physical interaction of the transcriptional apparatus with the template in this region.

TABLE 2. Transcriptional competence of LS mutants surrounding the HSP

Clone	Level of transcription[a]	
	LSP	HSP
LS 501/511	100	100
LS 506/514	100	100
LS 510/517	95	100
LS 517/524	104	106
LS 521/529	97	3
LS 526/538	100	1
LS 536/547	110	1
LS 543/551	95	1
LS 552/561	100	0
LS 558/566	100	0
LS 565/573	95	95
LS 572/581	105	99
LS 578/589	100	107
LS 588/597	105	100
LS 601/608	100	102

[a] Expressed as in Table 1.

All LS mutants upstream of nt 445 were completely wild type in LSP activity. Thus, the mutants presented here defined a maximal region of approximately 50 bp, from nt 396 to 446, that was both necessary and sufficient for accurate and efficient initiation from the LSP in vitro. The HSP that was present on the same DNA fragment in all the LSP LS mutants tested showed little or no effect attributable to the LSP mutations described, with the possible exceptions of mutants 421/429, 439/449, and 435/444; these displayed some increase in the amount of HSP transcription. This finding was completely consistent with the observation of Fisher et al. (22) that mtTF1 interacts with both promoters. These mutations may have impaired the ability of the LSP to sequester transcription factor, thus increasing the mtTF1 pool available to interact with the HSP in the in vitro system.

Scanning of the HSP. The results of transcription of the LS mutants surrounding the HSP (Fig. 3) differed in a number of ways from results for those surrounding the LSP. The most significant difference was the fact that the mutants displayed fewer distinct phenotypes (Table 2). Besides the mutants that were wild type in activity, the linker substitutions displayed either a minimal level of transcription (i.e., less than 5%, detectable only at high extract-to-template ratios, as demonstrated by 521/529, 526/538, 536/547, and 543/551) or no detectable transcription (552/561 and 558/566). (Note

that the approximately 5% level of HSP transcription was not visible in the exposure shown.) These results were in contrast to those obtained for the LSP mutations described above, which produced a greater number of intermediate phenotypes.

The disparate response to LS mutation by the HSP versus the LSP may reflect one or more of the following. The selection of LS mutants around the HSP may have resulted in mutations that altered so many critical bases that no intermediate phenotypes were observed. In support of this idea was the fact that no strict analog (in terms of position with respect to the start site) to LSP LS mutant 411/420 or 428/436, each of which appeared to fall between critical domains of the LSP and was intermediate in transcriptional deficits, was present in the HSP LS mutants. Although this possibility cannot be ruled out, a simpler explanation may be that the HSP is more sensitive to small perturbations in its sequence (25). It is tempting to speculate that the HSP (which has been shown to be more variable in ability to be transcribed in vitro [20], displays a less distinct mtTF1 footprint [22], and has been shown to bind mtTF1 less stably in DNA-binding kinetic experiments [21]) may be more sensitive to LS mutations as a result of its more tenuous binding of mtTF1. Any significant alteration in sequence may lower the ability of the mtTF1 to bind to such an extent that transcription from the HSP is reduced to background levels. The LSP, with its apparent higher affinity for mtTF1, could, as a result, be more resistant to mutations which lower the affinity for mtTF1.

As a whole, the results from the HSP mutagenesis are in excellent agreement with those of previous studies (6, 12). Chang and Clayton (12) found that deletion to −16 of the HSP start site allowed detectable starts but that further deletion eliminated transcription. Four LS mutations (521/529, 526/538, 536/547, and 543/551) did appear to allow transcription to initiate, but at an extremely reduced level only detectable at very high extract-to-DNA ratios. These mutants thus seem to delineate an upstream region that, when altered, profoundly affects the efficiency of initiation while permitting production of some low level of accurately initiated transcripts. Mutants 552/561 and 558/566 produced no detectable full-length HSP transcripts under any conditions tested. These mutations all altered multiple nucleotides immediately surrounding the start site itself. Thus, as with the LSP, there seemed to be a small region encompassing the HSP start site that was absolutely required for any level of initiation. These results allowed us to pare down the minimal promoter (defined as those sequences required for any level of accurately initiated transcription in vitro) to approximately nt 552 to 566 and to implicate sequences from nt 523 to 566 as being necessary for a wild-type level of transcription. None of the HSP-proximal LS mutants had any detectable effect on LSP transcription.

Analysis of HPM. By virtue of its asymmetry, the consensus mtTF1-binding-site sequence proposed by Fisher et al. (22) raised an obvious question with respect to the two start sites at the LSP and HSP: can or does the transcriptional apparatus operating at these promoters act bidirectionally? The data of Chang et al. (16) showed a low level of oppositely oriented transcription originating from both the LSP and the HSP. Furthermore, the mitochondrial promoters of *Xenopus laevis* have been shown to function bidirectionally in vitro (9).

In an attempt to address this question, we constructed mutants with rearranged promoter sequences (termed hybrid promoter mutants, or HPM). The strategy (Fig. 4A) consisted of isolating the LSP start site and placing the mtTF1-binding site of the HSP upstream of it. This was accomplished by taking the LSP mutant deleted to nt 410, which included the LSP start site at nt 407 and the *Bam*HI linker (essentially one half of the LS 411/420 mutant) and joining it with a deletion mutant containing some part of the HSP upstream region; in this case, the HSP mutant deleted to nt 518, which contained the HSP start site at nt 559 and upstream sequences to nt 518. The result was a mutant with both start sites intact but nt 411 to 517 deleted and a *Bam*HI linker inserted in its place (designated HPM 411/517). In this particular case, the result was that the LSP start site was brought into proximity with the HSP upstream region containing its mtTF1-binding site. These particular deletions were chosen because we reasoned that the LSP deletion to nt 410 retained the ability to initiate transcription when juxtaposed with a suitable upstream region (see above), albeit at a reduced level, whereas the deletion to nt 518 seemed to contain all sequences necessary for HSP transcription. Thus, a trimmed LSP start site was recombined with an intact HSP upstream region and its cognate start site.

When this mutant was assayed for ability to be transcribed in vitro, it displayed a significant level of LSP transcription (Fig. 4B). The two essential control templates for this experiment were the simple deletion to nt 410 [pKB 410(Δ411-741)] and the LS 411/420 mutant, which has wild-type LSP sequences upstream of the linker at nt 411 to 420. The simple deletion displayed no detectable LSP transcription, whereas the HPM 411/517 mutant was comparable to the LS 411/420 mutant in ability to support LSP transcription (Fig. 4B). Thus, the insertion of vector sequences upstream of the LSP start site in this construction allowed no LSP transcription, whereas the juxtaposition of either the wild-type LSP or HSP upstream sequences resulted in a significant level of accurate initiation from the LSP start site. The fact that the level of transcription supported by this HPM mutant was essentially the same as that of the parental LSP-LS 411/420 mutant argued that the transcriptional apparatus was operating in a fairly efficient manner. Furthermore, transcription from the correct HSP start site, present only 57 nt away from the LSP start site in this construction and presumably under the control of the same upstream element that was modulating L-strand transcription in this instance, was of wild-type efficacy. The presence of transcripts initiating from both the LSP and HSP start sites in this construct was dependent on both the mtRNA polymerase itself and other factors present in the extract. This conclusion was demonstrated by transcribing this HPM mutant with extracts that contained mtRNA polymerase activity but had been depleted of other factors required for selective transcription from the promoters (22). Under these conditions, very little specific transcription was observed unless the reactions were supplemented with extracts from which these factors had not been depleted (data not shown). HPM mutant 411/517 thus appeared to be an example of one upstream element, the HSP upstream region, promoting accurate transcription from two oppositely oriented start sites, positioned on either side of this putative control signal. Presumably, the mtTF1 bound in this region, and possibly other factors present, were able to act bidirectionally.

Interestingly, three other HPM mutants constructed analogously to HPM 411/517 (411/524, 421/517, and 421/524) were all unable to promote any LSP transcription (Fig. 4; data not shown), although they were all wild type with respect to the HSP, as expected. In Fig. 4C, the sequences of two of the HPM mutants are aligned with respect to the

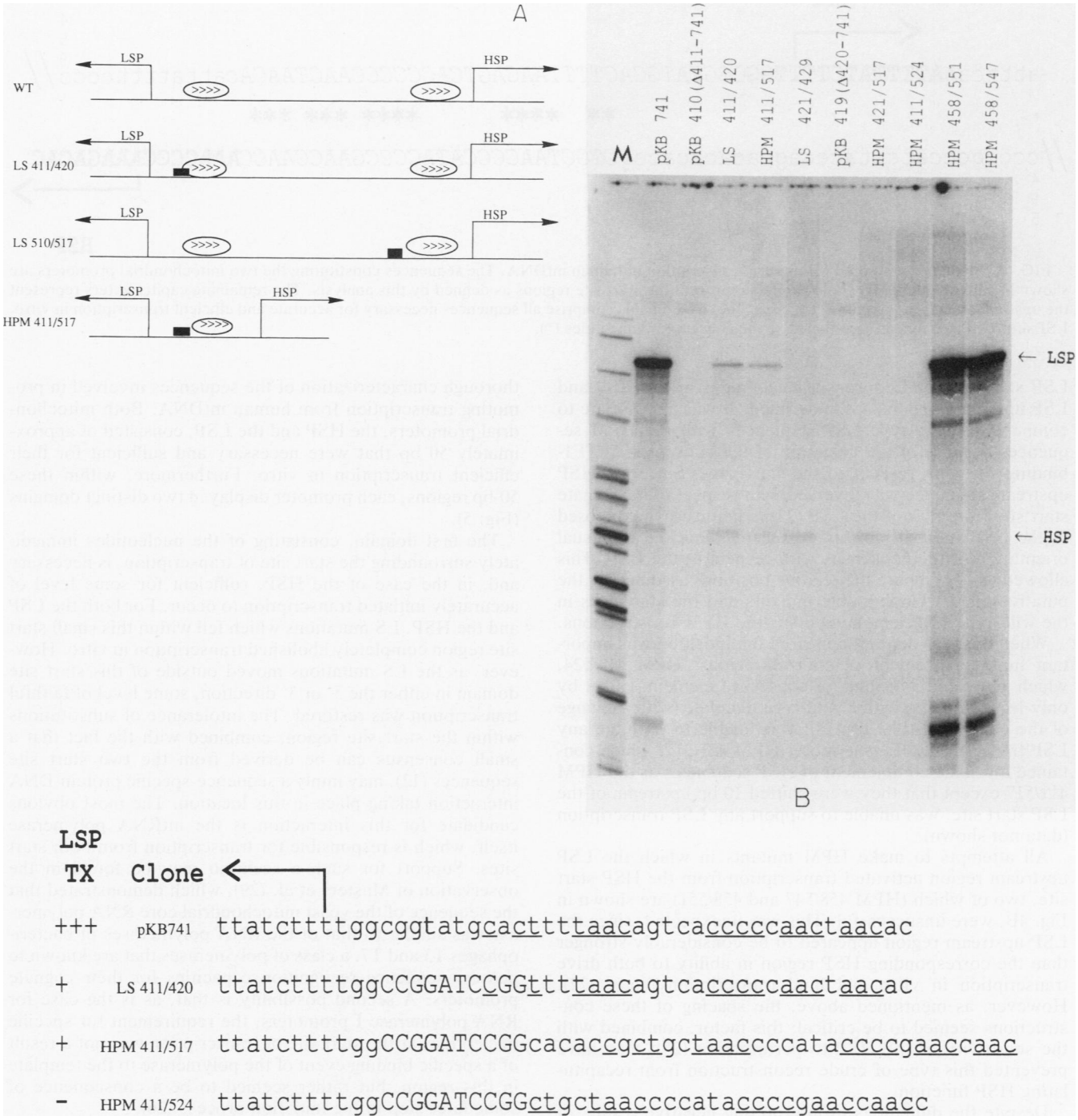

FIG. 4. Analysis of hybrid promoter mutants. (A) Schematic diagrams illustrating construction of hybrid promoter mutant HPM 411/517. The top line represents the wild-type (wt) clone pKB741, with both promoters indicated by a start site (arrow) and an mtTF1-binding site (oval). LS 411/420 and LS 510/517 are LS mutants described in Fig. 2 and 3, respectively. Black boxes denote approximate positions of the *Bam*HI linker. HPM 411/517 was constructed by combining sequences to the left of the linker in LS 411/420 with sequences to the right of the linker in LS 510/517. The HPM mutants, like the LS mutants, are designated by the nucleotides that have been deleted and replaced by the *Bam*HI linker. See text for details. (B) Autoradiogram of in vitro transcription of the HPMs. M, *Hpa*II-cleaved pBR322; pKB741, wild-type template; pKB410(Δ411-741), a deletion clone of pKB741 containing nt 1 to 410 of mitochondrial sequence with a *Bam*HI linker. pKB419(Δ420-741) contains nucleotides 1 to 419 of mitochondrial sequence with a *Bam*HI linker. Positions of the expected LSP and HSP runoffs are indicated. These should not vary for any of the constructions, since nothing distal to the respective start sites has been altered. (C) Sequences and LSP transcriptional phenotypes of pKB741, LS 411/420, HPM 411/517, and HPM 411/524. Only the sequences surrounding the start site of transcription of the LSP are shown. These are aligned at the start site itself. The *Bam*HI linker present in the LS and HPM mutants is in uppercase, and conserved nucleotides present in both the LSP and HSP upstream regions are underlined. See text for details. Transcriptional (TX) phenotypes: +++, wild type; +, 5 to 30% of wild type; −, no detectable transcription.

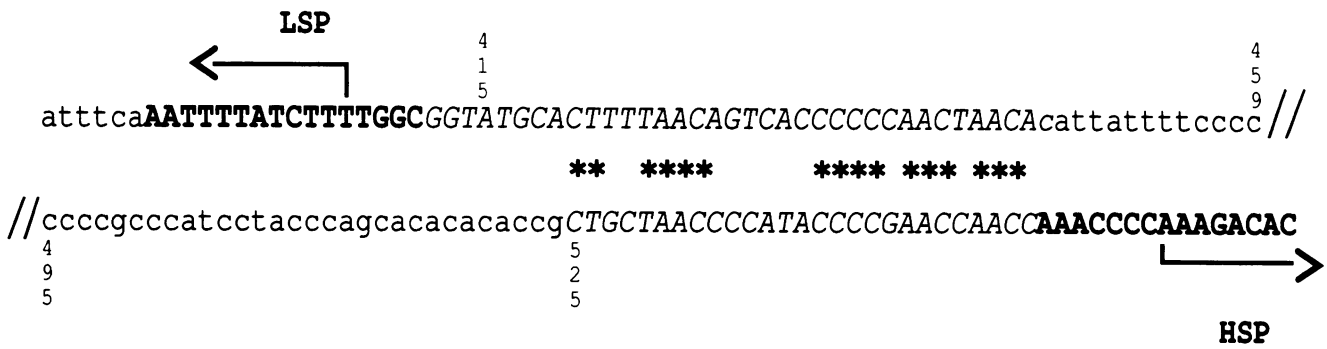

FIG. 5. Sequences required for in vitro transcription of human mtDNA. The sequences constituting the two mitochondrial promoters are shown in capital letters. The bold capitals represent the start site regions as defined by this analysis. The remaining capital letters represent the upstream regulatory regions. Together, the two domains comprise all sequences necessary for accurate and efficient transcription in vitro. LSP and HSP sequences are aligned at the conserved nucleotides (*).

LSP start site, and consensus nucleotides of the HSP and LSP upstream regions are underlined. It was appropriate to compare the wild-type LSP sequences with the HPM sequences because of the inversion of the consensus mtTF1-binding site with respect to the start sites. Since the HSP upstream sequence was reversed with respect to its cognate start site from that of the LSP (22), when it was juxtaposed to the LSP start site in this manner it mimicked the usual orientation of the consensus with respect to the LSP. This allowed us to compare the relative positions assumed by the putative mtTF1-binding determinants and the start sites in the wild-type LSP template versus the HPM constructions.

When this was done, it appeared that position was important in the placement of control regions. HPM 411/524, which generated a spacing of conserved elements offset by only 1 nt compared with a wild-type template (with a change of the conserved C at nt 418), was unable to promote any LSP transcription. Furthermore, HPM 421/517, which contained the same complement of HSP sequences as did HPM 411/517 except that they were shifted 10 bp upstream of the LSP start site, was unable to support any LSP transcription (data not shown).

All attempts to make HPM mutants in which the LSP upstream region activated transcription from the HSP start site, two of which (HPM 458/547 and 458/551) are shown in Fig. 4B, were unsuccessful. This was unexpected, since the LSP upstream region appeared to be considerably stronger than the corresponding HSP region in ability to both drive transcription in vitro and bind transcription factor (22). However, as mentioned above, the spacing of these constructions seemed to be critical; this factor, combined with the sensitivity of the HSP to perturbation (25), may have prevented this type of crude reconstruction from recapitulating HSP function.

Despite the difficulties in interpreting negative results of experiments such as these, the fact remains that the positive result obtained with HPM 411/517 was evidence that a single mitochondrial upstream promoter region can operate bidirectionally to promote accurate transcription from distinct, oppositely oriented start sites.

DISCUSSION

Sequence requirements for human mitochondrial promoters in vitro. The comprehensive mutagenesis presented here, together with the deletion analysis of Chang and Clayton (12), the point mutagenesis of Hixson and Clayton (25), and the biochemical analyses of Fisher et al. (20–22), allowed a thorough characterization of the sequences involved in promoting transcription from human mtDNA. Both mitochondrial promoters, the HSP and the LSP, consisted of approximately 50 bp that were necessary and sufficient for their efficient transcription in vitro. Furthermore, within these 50-bp regions, each promoter displayed two distinct domains (Fig. 5).

The first domain, consisting of the nucleotides immediately surrounding the start site of transcription, is necessary and, in the case of the HSP, sufficient for some level of accurately initiated transcription to occur. For both the LSP and the HSP, LS mutations which fell within this small start site region completely abolished transcription in vitro. However, as the LS mutations moved outside of this start site domain in either the 5′ or 3′ direction, some level of faithful transcription was restored. The intolerance of substitutions within the start site region, combined with the fact that a small consensus can be derived from the two start site sequences (12), may imply a sequence-specific protein-DNA interaction taking place in this location. The most obvious candidate for this interaction is the mtRNA polymerase itself, which is responsible for transcription from both start sites. Support for such a scenario may be found in the observation of Masters et al. (29), which demonstrated that the sequence of the yeast mitochondrial core RNA polymerase was similar to that of the RNA polymerases of bacteriophages T3 and T7, a class of polymerases that are known to possess sequence recognition capacities for their cognate promoters. A second possibility is that, as is the case for RNA polymerase I promoters, the requirement for specific nucleotides at the start site of transcription was not a result of a specific binding event of the polymerase to the template in this region, but rather seemed to be a consequence of some later step in the initiation process (26).

A distinction between the two start site domains becomes apparent when one considers whether this start site region alone is sufficient for transcription to occur. This is almost certainly the case for the HSP start site, as demonstrated by Fisher et al. (22) and implied by the response of the HSP to linker substitution analysis. The LSP start site domain, in contrast, lacked the ability to initiate accurately in the absence of specific sequences upstream of nt 421 (−14 of the start site). This was evidenced both by the 5′ deletion to nt 420 and by the HPM mutants that were constructed from this deletion, all of which were completely unable to support any detectable LSP transcription in vitro. This finding raised the possibility that sequences extending into the LSP upstream

domain (see below) were required for LSP transcriptional initiation.

The second promoter domain consisted of the upstream nucleotides of both the HSP and the LSP from approximately −10 to −40 of the start sites. This upstream element modulated the efficiency of transcription at both the LSP and the HSP. In each case, substitutions within this sequence lowered the efficiency of transcription while sparing the ability of the template to support accurate initiation. However, as with the start site domain, there were some important differences between the HSP and the LSP in the characteristics of the upstream domains.

The HSP upstream domain was quite sensitive to mutation. Any linker substitution which caused multiple changes within this region of the HSP produced a dramatic drop in the efficiency of transcription to levels comparable to that displayed by the start site alone. The LSP, on the other hand, showed a more varied response to mutation in this region. The LS mutations of the LSP upstream domain effected a number of intermediate phenotypes, ranging from a very low level of transcription to approximately 50% of wild type. Linker scanning of this region indicated that the upstream domain of the LSP may itself consist of a number of subdomains.

Previous data demonstrated the functional importance of the binding of a common transcription factor, mtTF1, to this region of both promoters (22). For the LSP LS mutants, there was a clear correlation between the transcriptional deficits of mutants in this region and their ability to bind the transcription factor stably in vitro; mutations in this region of both promoters appeared to disrupt the productive binding of mtTF1. The simplest model for both promoters is one in which mtTF1 is interacting with the template in the upstream domain, thereby increasing the efficiency of initiation of a weakly sequence-specific RNA polymerase. However, as previously mentioned, the HSP upstream domain has a lower affinity for mtTF1 in vitro (21). In addition, the orientation of the mtTF1-binding site with respect to the major directions of transcription for the LSP is opposite that for the HSP (22). These two facts may account for many of the differences in the wild-type behavior of these promoters in vitro as well as for their response to linker substitution.

Despite the contrasts between the two promoters, it was clear from this analysis that a general model may be posited. Its essence consists of a specific sequence around the start site of transcription that is required for any initiation to occur and which may specifically interact with the RNA polymerase molecule, combined with an upstream regulatory domain capable of binding a transcription factor and thereby modulating the efficiency of transcription; these features are sufficient to account for virtually all of the observations presented (Fig. 5).

Bidirectionality of the upstream domain. The conserved sequences present in the upstream domains of the human mitochondrial promoters occur in opposite orientations with respect to the major directions of transcription of the HSP and the LSP, respectively. This observation, coupled with the demonstration that a single species of mitochondrial transcription factor could bind to these regions and stimulate transcription from both promoters (22), implied that this upstream domain may act bidirectionally. The results of transcription of the hybrid promoter mutant HPM 411/517 provided evidence for this; the mutant contained a single HSP upstream domain flanked by both the LSP and HSP start sites and supported transcription accurately and efficiently at both sites. Therefore a single asymmetric sequence element seems able to stimulate transcription in an orientation-independent manner. Furthermore, a single upstream domain can apparently activate two divergently arranged transcriptional start sites present in a single construct (any requirement for the HSP start site domain in activating the LSP remains to be tested). This mitochondrial promoter upstream site, or mtTF1 responsive element, seems to be a member of a small group of transcriptional regulatory sequences, such as those acting at the nuclear factor I-binding site (19), the simian virus 40 21-bp repeats (2, 23), and the adenovirus type 2 major late promoter upstream element (28), which are able to operate bidirectionally. In contrast to its orientation independence, this upstream element may be quite position dependent, as evidenced by the failure of most of the hybrid promoter constructions to reconstitute active promoters.

Do mitochondrial promoters have a common organization? Vertebrate mitochondrial genomes are similar in overall organization of control elements, such as those regulating DNA replication and transcription, yet they share limited amounts of primary sequence homology in these regions (18). This characteristic seems to be reflected in the fine structure of their promoters as well. The basic organization of the murine (13–15) and *Xenopus* (7–9) mitochondrial promoters is reminiscent of the organization of human promoters; in general, it consists of a start site domain responsible for initiation (the murine HSP may be an exception (15)) and flanking regions that may control the rate of initiation. Despite the overall organizational similarity of these other vertebrate systems to the human paradigm, important differences exist. There is essentially no sequence homology between the promoter sequences of the three species, and heterologous experiments involving the enzymes of one species and the templates of another have not demonstrated specific transcription. Furthermore, a number of differences in behavior in vitro, including an apparent lack of a requirement for specific nucleotides at the start site of transcription of the murine HSP (15) and significant levels of bidirectional transcription from closely spaced start sites observed in *X. laevis* (8, 9), have been reported.

Standing in contrast to their vertebrate counterparts, the promoters of *Drosophila* mtDNA are likely different in organization (17). This genome lacks the characteristic D-loop structural features common to vertebrate genomes. Instead, there exists an extensive A+T-rich region which may contain the control elements for transcription and mtDNA replication. Therefore, although currently the transcription system is not well characterized, it promises to be distinct from that of the vertebrates.

The yeast *Saccharomyces cerevisiae* possesses a circular mitochondrial genome of approximately 75 kb. Yeast mtDNA promoters consist of a conserved nonanucleotide sequence from −8 to +1 of the start site of transcription, as shown by both deletion mutagenesis and analysis of the effects of point mutations on transcriptional initiation in vitro (4, 5). This is one of the smallest promoter sequences to be defined in any system and is obviously different from the more complex multidomain vertebrate mitochondrial promoters. Recently, a specificity factor for yeast mtRNA polymerase has been described that confers, to a core RNA polymerase, the ability to recognize mitochondrial promoters (34). The functional mode of this factor seems to resemble the action of sigma factor on *Escherichia coli* RNA polymerase rather than a template-binding mechanism.

Are mitochondrial promoters related to nuclear promoters? A growing body of data is accumulating which suggests that

the eucaryotic RNA polymerases and their cognate promoters have evolved from procaryotic ancestors. The demonstrations of significant identities between the sequences of the β′ subunit of *E. coli* RNA polymerase and the largest subunits of RNA polymerases II and III (1, 3), as well as identities between the conserved promoter elements such as the TATA sequences of both procaryotes and eucaryotes (30), lend considerable support to this hypothesis. Mitochondrial promoters are no exception to this rule, since they display similarities to both procaryotic and eucaryotic nuclear promoters. The yeast mitochondrial promoter exhibits considerable homology with the Pribnow box of procaryotic promoters (5), and its transcriptional machinery may consist of an amalgamation of bacteriophage and *E. coli* features in its mechanism of promoter recognition (29, 34). The human mitochondrial promoters (and possibly vertebrate mtDNA promoters in general) have a more complex promoter structure that involves the sequence-specific interaction of a separable transcription factor with a functional domain distinct from the site of polymerase action.

Of the three prototypical eucaryotic nuclear promoters, human mitochondrial promoters seem to resemble most those recognized by RNA polymerase I (35, 36). They both consist of approximately 50 to 60 bp immediately surrounding and upstream of the transcriptional start sites. Furthermore, both promoters are capable of forming preinitiation complexes that can sequester essential transcription factors (22, 27, 35). However, the polymerase I situation is distinct from and somewhat more complex than that of the mitochondrial promoters, since sequences up to approximately 100 bp upstream of the start site can be shown to influence transcription and may act as RNA polymerase I-specific enhancers (35). We note that any role for additional mtDNA sequences in transcription in vivo has not been tested; development of a transformation system for vertebrate mitochondria may permit such an analysis.

Although some similarities to the simplest promoters of RNA polymerase II are present, in general the polymerase II promoter is significantly more complex and physically larger than those of human mitochondria (10). Similarly, the mitochondrial promoters display no significant intragenic regions characteristic of genes transcribed by RNA polymerase III (33) and some polymerase II genetic units (10).

Thus, the mitochondrial promoters of humans, and perhaps all vertebrates, constitute a unique class of transcriptional regulatory sequences. However, in view of the recent example of sequence elements common to promoters of polymerases II and III interacting in the transcription of U6 (11) and possibly the 7SK gene (32), the distinctions between these transcriptional classes are being blurred. It will be interesting to learn whether any sequence elements or factors are shared between the nuclear and mitochondrial transcriptional apparatus.

ACKNOWLEDGMENTS

We thank D. D. Chang and R. P. Fisher for reviews of the manuscript.

This investigation was supported by Public Health Service grant GM33088-18 from the National Institute of General Medical Sciences and grant NP-9N from the American Cancer Society, Inc. J.N.T. is a Medical Scientist Training Program trainee of the National Institute of General Medical Sciences (Public Health Service grant GM07365-12).

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