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23. The gene-specific probes used to probe RNA blots were isolated from low melting point agarose gels after digestion of the plasmids in which they were propagated with appropriate restriction endonucleases. Run-on transcription assays were performed with plasmids containing gene-specific inserts denatured by alkali treatment and transferred to nitrocellulose filters in a slot blot apparatus. The IL-2 probe was a 1.0-kb Pst I cDNA cloned into pBR322 (provided by S. Arya). The IFN-γ probe was a 1.0-kb Pst I cDNA fragment cloned into pBR322 (obtained from H. Young). The TNF-α probe was a 0.5-kb Eco RI–Bam HI cDNA fragment (provided by S. Kunkel). The GM-CSF probe was a 0.7-kb Eco RI–Hind III fragment (provided by S. Emerson). The 4F2HC probe was a 1.9-kb Eco RI cDNA fragment in pUC18 (provided by J. Leiden). The HLA probe was a 1.4-kb Pst I fragment from the HLA-B7 gene in pGEM-4. The c-*fos* probe was a 0.9-kb Sca I–Nco I fragment of pc-*fos*-1 available from the American Type Culture Collection. The c-*myc* probe was a 1.0-kb Cla I–Eco RI fragment isolated from a human c-*myc* cDNA (provided by D. Bentley). The IL-2 receptor probe was a 1.9-kb Eco RI–Bam HI fragment specific for the 55-kD IL-2 receptor subunit derived from pIL-2R3 (obtained from W. Greene).
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The Role of Cis-Acting Promoter Elements in Tissue-Specific Albumin Gene Expression

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The mouse albumin gene promoter has six closely spaced binding sites for nuclear proteins that are located between the TATA motif and nucleotide position −170. In vitro transcription with liver or spleen nuclear extracts of templates containing either mutated or polymerized albumin promoter elements establishes a hierarchy of the different protein binding sites for tissue-specific albumin gene transcription. The HNF-1 and C/EBP binding sites strongly activate transcription in a tissue-specific manner. The NF-Y binding site has a lower activation potential and is less specific, being equally efficient in liver and spleen nuclear extracts. The remaining elements are relatively weak activator sites.

THE ACTIVITY OF MOST TISSUE SPECIFICALLY expressed mRNA genes is controlled primarily at transcription initiation (*1*). Three types of cis-acting elements may regulate the efficiency of this process: chromatin openers (*2*), enhancers (*3*), and promoters (*4*). Chromatin openers may decondense chromatin domains from a repressed to a potentially active state, thus making the gene or genes accessible to the transcription machinery. Enhancers can increase transcription initiation frequency from a distance in a location- and orientation-independent manner. Promoters are bipartite structures consisting of a core promoter and an upstream regulatory region. The core promoter (generally a TATA box, or equivalent element, and cap site) guides RNA polymerase II to the correct start site. The upstream regulatory region comprises one to several elements that can augment or decrease initiation frequency. Structural and functional studies on many promoters and enhancers have indicated that both have a modular structure and that some modules can be shared between the two. Both may therefore be assembled into a preinitiation complex that can be recognized efficiently by the transcription machinery, and this assembly may be mediated by trans-acting transcription factors that recognize specific DNA sequence elements with one functional domain and general components of the transcription apparatus such as RNA polymerase II and factor TFIID with another domain (*5*).

The serum albumin gene may need tissue-specific chromatin opener, enhancer, and promoter elements for its developmental activation during liver differentiation (*6*). In transient transfection studies, however, a relatively short 5′ flanking segment of about 150 nucleotides was sufficient to direct hepatocyte-specific transcription (*7*). We have also found that this sequence is necessary and sufficient to confer tissue-specific in vitro transcription to a G-free cassette (synthetic DNA fragment that does not contain any guanosine residue in its noncoding strand) reporter gene (*8*). As our in vitro system consists of histone-free nuclear extracts (NEs) and plasmid DNA templates, this differential in vitro transcription does not seem to need complex chromatin structures; more likely, it is the direct result of trans-acting tissue-specific transcriptional activators. We therefore established an inventory of DNA binding proteins that interact with albumin gene promoter elements, both for liver, the expressing tissue, and spleen, a nonexpressing tissue (*9–11*). In agreement with the results of other groups (*12*), the identified DNA binding proteins include the liver-specific or liver-enriched factors HNF-1 (*13*), C/EBP (*14*) and DBP (*10*), several CTF/NF-1–related factors (*15*), and the ubiquitous CAAT-factor NF-Y (*16*) (Fig. 1).

Results are now presented of two experimental strategies that were chosen to evaluate the relative importance of the protein-binding promoter elements in conferring liver-specific transcription of the albumin gene. In the first approach each individual binding site [with the exception of site F, whose deletion had only a marginal impact on transcription (*8*)] was destroyed by a short substitution of 11 or 13 nucleotides with unrelated DNA (Fig. 2A). The resulting templates contained the mutated promoters PΔA to PΔE, each having lost the capacity to interact with the cognate factor for the mutated site (*17*). The mutagenized templates, together with templates containing the wild-type promoter (P_{wt}) and the core promoter (PΔ-35, which consists of the TATA motif and the cap site) were subjected to transcriptional analysis with liver and spleen NEs (Fig. 2B).

In each in vitro transcription reaction, AdML200, a template containing the adenovirus major late promoter, was used as an internal standard. This promoter is recognized with a similar efficiency in both liver and spleen NEs (*8*). The relative transcriptional potential of the albumin promoter in liver and spleen was evaluated by dividing the radioactivity associated with the albumin transcript by the one associated with the adenovirus major late transcript. This ratio suggests that the albumin promoter is approximately 50-fold as active in liver as in spleen.

In liver NE, substitution of site B had the greatest effect on transcription, reducing the transcription efficiency approximately tenfold. In contrast, this same mutation had no significant effect on transcription in spleen NE. This result is consistent with that of a binding assay (Fig. 3B), which demonstrates that the cognate factor for this site, HNF-1, is liver-specific. In addition to the HNF-1 complex, a number of less abundant, more rapidly migrating complexes were observed with both liver and spleen

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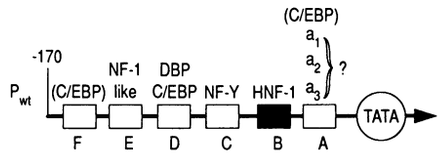

Fig. 1. Schematic representation of the DNA binding factors interacting with the albumin promoter. Most of the transcription factors binding to albumin promoter elements have been identified. Element A appears to have affinity to multiple proteins, some of which (a_1, a_2, a_3) remain to be identified.

NEs. Some of these may be due to vHNF, a variant form of HNF-1 (*18*). We do not believe that vHNF binding activities contribute significantly to transcriptional activation because substitution of element B has no significant effect on in vitro transcription from the albumin promoter in spleen extracts and artificial promoters containing multimeric B elements are inactive in spleen NE (see below).

The substitutions within sites D and C resulted in a significant reduction of in vitro transcription in both liver and spleen NEs (Fig. 2B). These two sites alone may account for the residual albumin transcription in the spleen NE. The elimination of any other site has little, if any, effect on albumin in vitro transcription in spleen NE. The mutation of element C, the binding site for the ubiquitous CAAT-factor NF-Y, is sufficient to reduce the transcription signal in spleen NE to the background level observed with the core promoter PΔ-35.

The mutation of element E, a binding site of CTF/NF-1–related factors, resulted in a less than twofold reduction of transcription in liver NE. This suggests a minor role for these factors in the activation of albumin transcription in vitro. The relative importance of site E (and also of site C) for in vitro albumin transcription is considerably higher with templates propagated in *dam*$^+$ (deoxyadenosine methylase) bacterial strains (*9*), as compared to the templates grown in the *dam*$^-$ strain used in this work. This difference is due to the presence of a *dam* methylation site (GATC) in element B; when methylated, this sequence binds HNF-1 with 1/30th the affinity (*11*). Thus, promoters with an impaired HNF-1 binding site may have an increased requirement for the transcription factors CTF/NF-1 and NF-Y.

We did not observe any mutations resulting in more efficient transcription initiation in either spleen or liver NEs (Fig. 2). This suggests the absence of a negative cis-acting element operating in our in vitro transcription system. The differential albumin in vitro transcription is more likely controlled by activators, such as HNF-1 and C/EBP, that are present at much higher amounts in liver NE than in spleen NE. This interpretation is confirmed by the approach described below.

In our second experimental approach, we examined the activation potential of each element separately. Each binding site was chemically synthesized, oligomerized by ligation, and inserted in front of the core promoter to produce homopolymer promoters. The templates containing such artificial promoters were then tested for transcription efficiency in liver and spleen NEs (Fig. 4).

In liver NE, all of the six elements (A to F) can enhance transcription to some extent when polymerized. In spleen NE, only the three homopolymer promoters $(C)_n$, $(D)_n$, and $(A)_n$ stimulate transcription above the background obtained with the core promoter. As expected from the mutation analysis, element B stimulates transcription most efficiently in liver NE but is completely silent in spleen NE. A single copy of it enhances transcription more than tenfold (*11*) in liver NE and the maximal activation potential (49 times) was reached with only two copies. Thus HNF-1, the liver-specific factor interacting with this site, may be the dominant effector molecule in directing liver-specific albumin transcription. The activity of $P(B)_2$ or $P(B)_5$ is at least 150-fold as high in liver

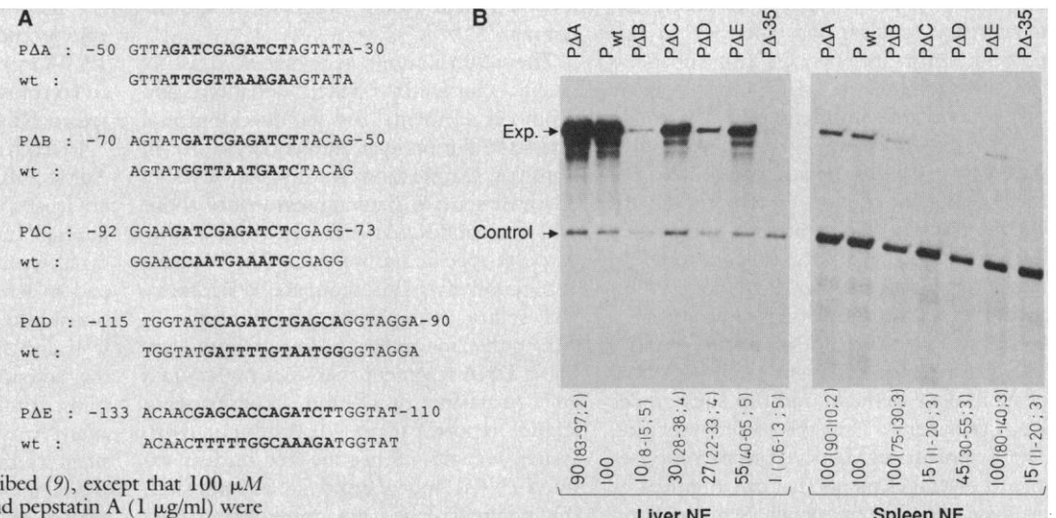

Fig. 2. In vitro transcription of templates with mutated protein-binding sites. (**A**) Binding sites A, B, C, D, and E of the template Alb400/−170 (wt) were mutated by oligonucleotide-directed mutagenesis (*9*). The sequence of the oligonucleotides and their boundaries are given together with their wild-type (wt) counterparts. The differences between the mutants (PΔ) and wt sequences are shown in bold letters. (**B**) Autoradiograms showing transcripts of templates with mutated promoters. Template (400 ng) containing albumin wt or site-mutated promoters was mixed with 200 ng of the control AdmL 200 template, and the mixture was incubated with liver or spleen NE (*8*). The NEs were prepared as described (*9*), except that 100 μ*M* benzamidine, leupeptin (1 μg/ml), and pepstatin A (1 μg/ml) were added to the homogenization and nuclear lysis buffers. The homogenization buffer for the isolation of spleen nuclei contained in addition 0.5% nonfat dry milk (an aqueous stock solution containing 1 g/10 ml was centrifuged by sedimentation at 10,000*g*, and 1 volume of the supernatant was then added in 20 volumes of homogenization buffer). The presence of milk proteins in the homogenization buffer increased the recovery of spleen nuclei and the transcriptional activity of these extracts. Transcription reaction mixtures (20 μl) contained liver nuclear proteins (4.5 mg/ml) or spleen nuclear proteins (2.8 mg/ml). Transcription reactions were performed as described (*8*) except that the reactions with spleen extracts contained 7 μ*M* uridine triphosphate and 0.5 m*M* 3′-*O*-methyl guanosine triphosphate instead of 35 μ*M* and 0.15 m*M*, respectively, with liver extracts. These adjustments were made to compensate for the greater contamination of spleen nuclei with free nucleotide triphosphates. The control plasmid AdML200 is a derivative of vector pML(C2AT)19 (*22*) with a shorter G-free cassette (200 nucleotides). Transcripts were analyzed on 5% sequencing gels. The regions containing the two ^{32}P-labeled transcripts were excised from dried gels, and suspended in aquasol (Amersham), and the radioactivity present was determined in a liquid scintillation counter. The transcriptional signals thus obtained were corrected for errors in sample handling by assuming that transcription for the plasmid AdML200 was equally efficient in each reaction. The results obtained with the mutated templates were expressed as a percentage of the value obtained for the Alb400/−170 (wt). The values can only be compared within each tissue, the albumin wt promoter being more active with liver than with spleen NEs. The percentage values are averages of several independent experiments. The minimum and the maximum values obtained and the number of experiments performed are stated in parentheses.

as in spleen NE. Therefore, these artificial promoters are even more liver-specific than the albumin P_{wt}.

Site D polymer promoters also show a high activity in liver NE but only at higher copy numbers: $P(D)_1$ and $P(D)_4$ activate transcription 6- and 25-fold, respectively. In accordance with the result obtained with the D mutant promoter (PΔD), this element can also activate transcription in spleen NE, albeit much less efficiently than in liver NE. This differential stimulation is consistent with binding studies showing that the cognate factor or factors of this site are much more abundant in liver than in spleen (*9, 10*).

Polymers of site C enhance transcription to a similar extent in spleen and liver NEs. In liver extracts $P(C)_7$ is considerably less potent than $P(B)_5$ or $P(D)_9$; however, in spleen extracts it is more active than any other promoter, including P_{wt}. This is consistent with the tissue distribution of the cognate CAAT-factor NF-Y. Among the various DNA binding proteins recognizing albumin promoter elements, NF-Y is the only transcription factor with a similar concentration in all tissues (Fig. 3C) (*9*).

The E and F polymer promoters increase transcription weakly in liver NEs and not at all in spleen. The tissue specificity of these weak promoters is compatible with their cognate factor distributions. There are many more NF-1–related species recognizing element E in liver than in spleen (*9*). Likewise, C/EBP, which binds element F with a low affinity, is highly enriched in liver. Some activation of transcription is also observed with $P(A)_4$ although elimination of this site had little, if any, effect on in vitro transcription. Site A is a low-affinity site for C/EBP (*9*) but also binds additional unidentified proteins (Fig. 3A).

None of the homopolymer promoters was as efficient in directing in vitro transcription in liver NE as the P_{wt}. Polymers of the strong activator sites B or D might have been expected to enhance transcription more efficiently than a composite of strong and weak activator sites. Thus, the precise location of each cis-acting element with regard to the transcription initiation site may be important, or the various factors may work cooperatively. We prefer the second explanation, since inversion of a promoter fragment containing binding sites B, C, D, E, and F has little effect on the promoter activity (*8*).

The results presented here establish a hierarchy of importance of the various cis-acting elements and their cognate factors for liver-specific in vitro transcription from the albumin promoter. The factors HNF-1, C/EBP or DBP, and NF-Y can independently activate transcription, probably by contacting one or several components of the basic transcription apparatus. The albumin promoter is thus composed of elements, which can by themselves reconstitute, when polymerized, either more liver-specific promoters [like $P(B)_n$ promoters)] or ubiquitous promoters [like $P(C)_n$ promoters]. Clearly, HNF-1 has a dominating role in controlling the liver-specific transcription of the albumin gene. The HNF-1 binding site appears to be the only albumin promoter element that was well conserved during vertebrate evolution (*19*). Furthermore, high-affinity recognition sites for this transcription factor have been identified in the promoters of many other liver-specific genes, including the one encoding α-fetoprotein (*20*), β-fibrinogen (*13*), and α1-antitrypsin (*21*). The effects of mutated albumin promoter elements have also been tested by transient transfection studies (*7*). Whenever comparable, the results obtained in our in vitro transcription system are in close agreement with those gathered from in vivo expression studies.

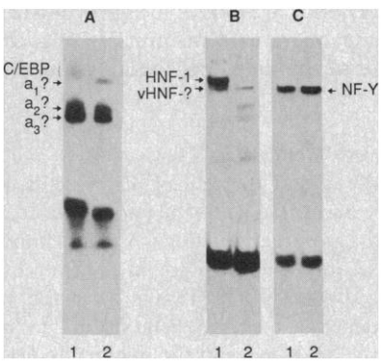

Fig. 3. DNA binding activities interacting with sites A, B, and C. Gel retardation assays with A (**A**), B (**B**), and C (**C**) single binding sites were performed essentially as described (*9*). Double-stranded deoxyribonucleotides of each site were end-labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]deoxyadenosine triphosphate and incubated for 10 min on ice with 5 μg of either liver (lanes 1) or spleen (lanes 2) NEs and 2 μg of double-stranded poly(dI-dC) competitor DNA in a final volume of 20 μl. The binding reaction mixtures were subjected to electrophoresis on a 6% nondenaturing polyacrylamide gel to separate free and retained DNA. The tissue distribution of factors that bind to elements D, E, and F has been demonstrated (*9*).

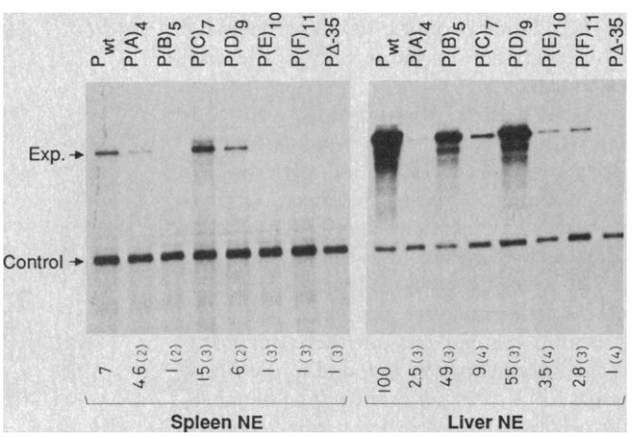

Fig. 4. In vitro transcription of templates with homopolymer promoters. Double-stranded deoxyribonucleotides containing a single binding site A to F were self-ligated before insertion at position −35 in the vector PΔ-35. This resulted in templates containing the homopolymer promoters $P(A)_n$ to $P(F)_n$ (*n* represents the number of each binding site). The sequence of each construct was confirmed by a G + A sequence reaction according to Maxam and Gilbert (*23*). The templates were mixed with the control template AdML200, and the mixtures were incubated as described in the legend to Fig 2. The transcription increase obtained with the different templates (below the gel) is expressed in multiples of the value (arbitrarily set as 1) obtained for PΔ-35. The values can only be compared within each tissue. The values are averages of several independent experiments (number in parentheses) and variability did not exceed ±30%. Several additional homopolymer promoters have been tested in liver NE. Their transcription efficiencies, as compared to the core promoter, are given in parentheses: $P(A)_2$ or $P(A)_6$ (2.5×); $P(B)_2$ (49×); $P(C)_3$ (3×); $P(C)_5$ (5×); $P(D)_1$ (6×); $P(D)_4$ (25×); $P(E)_1$, $P(E)_3$, or $P(E)_5$ (1.5×); and $P(E)_{14}$ (3.9×).

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A Direct Repeat Is a Hotspot for Large-Scale Deletion of Human Mitochondrial DNA

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Kearns-Sayre syndrome (KSS) and progressive external ophthalmoplegia (PEO) are related neuromuscular disorders characterized by ocular myopathy and ophthalmoplegia. Almost all patients with KSS and about half with PEO harbor large deletions in their mitochondrial genomes. The deletions differ in both size and location, except for one, 5 kilobases long, that is found in more than one-third of all patients examined. This common deletion was found to be flanked by a perfect 13–base pair direct repeat in the normal mitochondrial genome. This result suggests that homologous recombination deleting large regions of intervening mitochondrial DNA, which previously had been observed only in lower eukaryotes and plants, operates in mammalian mitochondrial genomes as well, and is at least one cause of the deletions found in these two related mitochondrial myopathies.

KEARNS-SAYRE SYNDROME (KSS) IS a multisystem mitochondrial disorder defined by the presence of ophthalmoplegia and pigmentary retinopathy with onset before age 20 and at least one of the following: high cerebrospinal fluid (CSF) protein content, blockage in heart conduction, or ataxia (*1*). Morphologically, KSS patients display "ragged red fibers" (RRF) in muscle sections; RRF are a morphologic hallmark of proliferating mitochondria in muscle and are seen in muscle sections stained with modified Gomori trichrome as red patches (*2*). KSS is ultimately fatal. Progressive external ophthalmoplegia (PEO) and, frequently, RRF are also seen in ocular myopathy, but there is no systemic involvement and the disease is rarely fatal. Biochemically, both KSS and PEO often show reduced respiratory chain enzyme activity, particularly that of cytochrome c oxidase (CO) (*3*, *4*).

We found that 13 of 15 patients with KSS and about half of all patients with PEO had large-scale deletions of mitochondrial DNA (mtDNA), ranging in size from 1.3 to 7.6 kb (*4*). Similar results have also been obtained by others (*5*). The size and location of the deletions, and the number of deleted mtDNA relative to the number of normal mitochondrial genomes, differed among patients and did not appear to be correlated to the presentation or the severity of the disease phenotype.

The 29 deletions we studied were mapped by analyzing each deleted genome for the absence of known restriction sites on the mtDNA map (*6*). Using this method, however, we were unable to specify the precise breakpoint of any deletion. Nevertheless, we showed that all the deletions were in regions of the mitochondrial genome containing structural components of the respiratory chain; no deletions were found in the ribosomal RNA genes, or in the region of either the origins of heavy- or light-strand replication, or of heavy- and light-strand transcription (*7*). Of the 29 deletions, 11 (3 in patients with KSS and 8 in patients with PEO) mapped to an identical location in the mtDNA, with deletion breakpoints about 5 kb apart, extending from the ATPase8 gene (*8*) of complex V on the left, to the gene encoding a subunit of NADH dehydrogenase (ND5) of complex I on the right. We have now found a 12th patient (with KSS) who also harbors this common deletion.

Using the polymerase chain reaction (PCR) (*9*), we amplified selectively the region of the deleted genome spanning the deleted mtDNA in a number of patients with this common deletion and determined the exact site of the deletion breakpoint by DNA sequencing (*10*, *11*).

We sequenced mtDNA from two patients with KSS and three patients with PEO who

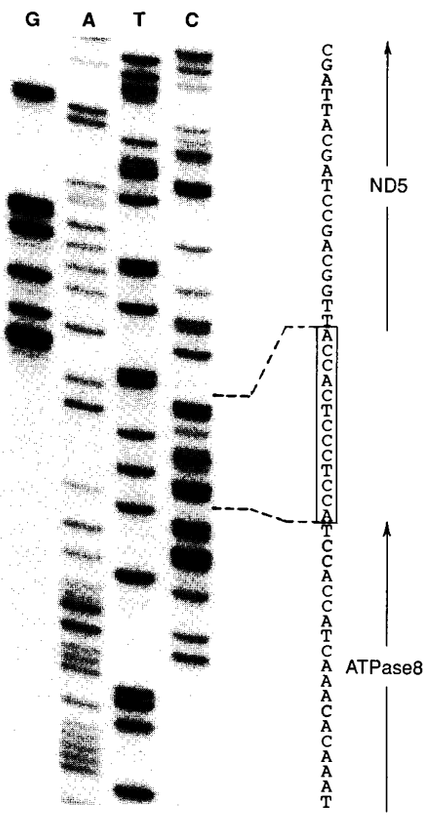

Fig. 1. Autoradiogram of a DNA sequencing gel in the region of the deletion breakpoint. The sequence (dideoxy reactions G, A, T, and C) is of the L-strand, reading 5′ to 3′ from bottom to top (arrows). The single 13-bp direct repeat (boxed) in the deleted mtDNA is downstream of the 5′ portion of the ATPase8 gene and upstream of the 3′ portion of the ND5 gene; the intervening 4977 bp are deleted (Fig. 2).

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