

**FIG. 3.** Fractionation of purified histones by FPLC. The proteins purified by the sequence of Cu-IMAC and Zn-IMAC were loaded onto a reverse-phase PEP-RPC column ( $C_2 + C_{18}$ ; purchased from Pharmacia LKB Biotechnology). Elution was obtained by delivering in 60 min a linear gradient between 0.1% v/v TFA in water and 0.08% v/v TFA in  $CH_3CN$ , at a flow rate of 0.5 ml/min. Main peaks are numbered 1–5.

tion, in the form of acetylserine, in histones H1, H2a, and H4 (5,6).

Since positive identification of all sample components was impossible by this method, further evidences were sought by less direct analytical approaches. The amino acid composition on the acid hydrolysate of the whole fraction (in mmol%) is as follows: D, 8.77; T, 5.51; S, 6.27; E, 11.77; G, 12.01; A, 10.01; V, 6.36; I, 4.52; L, 8.27; Y, 2.91; F, 2.46; K, 11.41; R, 9.73. The imbalance between acidic and basic amino acids points to alkaline  $pI$ 's for all components; this could be confirmed when trying to focus these proteins on immobilized pH gradients (7) and by running them on nonequilibrium pH gradient electrophoresis (8) (not shown). Taken together, these properties define the purified proteins as a whole histone fraction.

In conclusion, under the conditions described, IMAC is highly discriminative for histones against the background of a crude cell lysate. Typical purification protocols of whole nuclear proteins include nuclei fractionation by differential centrifugation, followed by acid extraction with perchloric or sulfuric acid (4). The procedure described in this paper, however, differed in that a *crude total cell lysate* was loaded onto IMAC columns, as sonication appears to disassemble the highly ordered nucleosome structure very efficiently (9). The proposed purification protocol is straightforward, the reagents are inexpensive, and the yield and purity of the pooled histones are very high.

## REFERENCES

1. Porath, J. (1990) *J. Mol. Recognit.* **3**, 123–127.
2. Jungblut, P., Baumeister, H., and Klose, J. (1993) *Electrophoresis* **14**, 638–643.

3. Aden, D. P., Fogel, A., Damjanov, I., Plotkin, S., and Knowles, B. B. (1979) *Nature* **282**, 615–616.
4. vonHolt, C., Brandt, W. F., Greyling, H. J., Lindsey, G. G., Retief, J. D., Rodrigues, J. de A., Schwanger, S., and Sewell, T. (1989) in *Methods in Enzymology* (P. M. Wassarman and R. D. Kornberg, Eds.), Vol. 170, pp. 431–523, Academic Press, San Diego.
5. Isenberg, I. (1979) *Annu. Rev. Biochem.* **48**, 159–191.
6. Liew, C. C., Haslett, G. W., and Allfrey, V. (1970) *Nature* **226**, 414–417.
7. Righetti, P. G., Delpech, M., Moisan, F., Krub, J., and Labie, D. (1983) *Electrophoresis* **4**, 393–398.
8. O'Farrell, P. Z., Goodman, H. M., and O'Farrell, P. H. (1977) *Cell* **12**, 1133–1142.
9. Noll, M., Thomas, J. O., and Kornberg, R. (1975) *Science* **187**, 1203–1206.
10. Laemmli, U. K. (1970) *Nature* **227**, 680–682.

## Identification of Human Mitochondrial DNA Fragments Corresponding to the Genes for ATPase, Cytochrome C Oxidase, and Nine tRNAs in a Denaturing Gradient Gel Electrophoresis System<sup>1</sup>

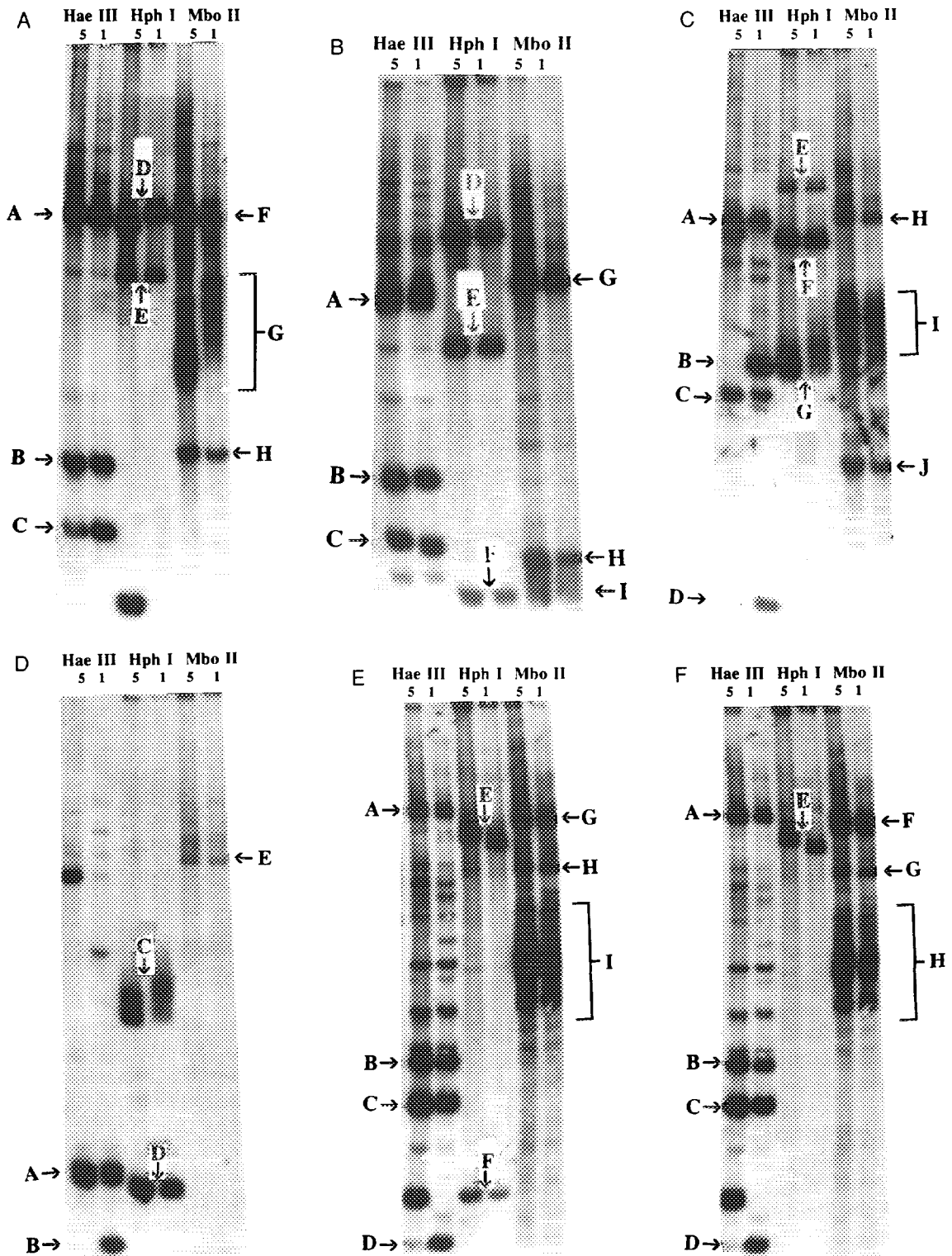
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A large and growing number of mitochondrial disorders have been associated with mtDNA mutations (1) and there have been suggestions of mitochondrial involvement in many other conditions. We recently reported a denaturing gradient gel electrophoresis (DGGE)<sup>2</sup> method for detecting small mutations in human mitochondrial DNA (mtDNA) (2). Point mutations, as well as insertions, deletions, and rearrangements too small to be detected by restriction fragment-length polymorphism analysis, can be identified. In this method mtDNA samples are digested with selected restriction enzymes and electrophoresed at 60°C through a 6.5% polyacrylamide gel containing a gradient of increasing denaturing concentrations of urea and formamide. A region of each DNA fragment will denature at a specific point in the gel. Denaturation greatly decreases fragment mobility. Two mtDNA molecules of the same size that differ by a single base pair in their first melting domains will have different final positions in the gel, referred to as melting behavior polymorphisms

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<sup>2</sup> Abbreviations used: DGGE, denaturing gradient gel electrophoresis; MBPs, melting behavior polymorphisms.



**FIG. 1.** Autoradiographs of mtDNA fragments separated by DGGE and hybridized with mtDNA probes 4-9. The probes were then removed from the blot and each blot was hybridized to purified restriction fragments to determine the identity of each band. The lettered bands in Figs. 1A-1F correspond to the lettered fragments in Table 1.

TABLE 1

Key to mtDNA Fragments Identified by Letters in the Denaturing Gradient Gel System, as Shown in Figs. 1A–1F

| Lane (enzyme)  | Fragments identified by letters in Figs. 1A–1F   |
|--|--|
| Fig. 1A, Probe #4 (nt 5275–6204: ND 2, tRNAs O <sub>L</sub> , COX I)<br><i>HaeIII</i><br><i>HphI</i><br><i>MboII</i>     | A, 5262–5838; B, 6028–6261; C, 5838–6028<br>D, 5315–5806; E, 5884–6612<br>F, 5283–5778; G, 5778–6095; H, 6095–6335                             |
| Fig. 1B, Probe #5 (nt 6204–7441: COX I)<br><i>HaeIII</i><br><i>HphI</i><br><i>MboII</i>                                  | A, 6384–6958; B, 7198–7498; C, 6958–7198<br>D, 6938–7639; E, 5884–6612; F, 6612–6809<br>F, 6551–7169; H, 6095–6635; I, 7169–7371               |
| Fig. 1C, Probe #6 (nt 7441–8287: COX I, II, tRNAs, ATPase 6,8)<br><i>HaeIII</i><br><i>HphI</i><br><i>MboII</i>           | A, 7498–7887; B, 7887–8251; C, 7198–7498; D, 8251–8392<br>E, 6938–7639; F, 7639–8128; G, 8128–8425<br>H, 7927–9093; I, 7649–7927; J, 7431–7649 |
| Fig. 1D, Probe #7 (nt 8287–8592: COX II, tRNA, ATPase 6,8)<br><i>HaeIII</i><br><i>HphI</i><br><i>MboII</i>               | A, 8392–8573; B, 8251–8392<br>C, 8128–8425; D, 8469–8646<br>E, 7927–9093   |
| Fig. 1E, Probe #8 (nt 8592–9648: COX II, III, ATPase 6,8, ND 3, tRNAs)<br><i>HaeIII</i><br><i>HphI</i><br><i>MboII</i>   | A, 9554–10,365; B, 8573–8839; C, 9026–9267; D, 8839–8995<br>E, 8646–9560; F, 8469–8646<br>G, 9476–10,214; H, 7927–9093; I, 9093–9476           |
| Fig. 1F, Probe #9 (nt 8729–10,254: COX II, III, ATPase 6,8, ND 3, tRNAs)<br><i>HaeIII</i><br><i>HphI</i><br><i>MboII</i> | A, 9554–10,365; B, 8573–8839; C, 9026–9267; D, 8839–8995<br>E, 8646–9560<br>F, 9476–10,214; G, 7927–9093; H, 9093–9476                         |

Note. Numbers refer to nucleotides in the human mtDNA sequence according to Anderson *et al.* (4).

(MBPs) (2,3). Because separation of molecules in this DGGE gel system is not predictable by size, it was important to identify the fragments of mtDNA that are detected in the DGGE gels to minimize the amount of sequencing required to pinpoint mutations detected by MBPs. The fragments studied were between nt 5274 and 10,254 (4). We chose this region because it contains the genes for important subunits of the major energy transducing complexes in the mitochondrial respiratory chain, as well as almost half of the mitochondrial tRNAs.

Human liver samples which had been stored for 3–8 years at  $-70^{\circ}\text{C}$  were remnants from surgical or autopsy specimens from individuals without any symptoms of mitochondrial dysfunction. MtDNA was isolated by standard procedures (2,5,6). Three separate digestions of 10–20 ng of mtDNA were carried out for 5 h with the restriction enzymes *HaeIII*, *HphI*, and *MboII*. The samples were run at 65 V in 30–80% DGGE systems for 18 h at  $60^{\circ}\text{C}$  followed by electroblotting onto a nylon membrane at 300 mA for 4 h (2,5,6). Mitochondrial probes 4–9 from Dr. G. Attardi covering nt 5275–10,254 were analyzed in this study (2). Each probe was purified, labeled with  $^{32}\text{P}$  using the Randon Primers DNA Labeling System (Gibco/BRL Life Technologies, Inc.), and hybridized to a Southern blot of the digested mtDNA. Each filter was then stripped and successively probed with

$^{32}\text{P}$ -labeled isolated restriction fragments corresponding to a specific region of the original probe.

Figures 1A–1F show the results of hybridizing probes 4–9 in their entirety with mtDNA from two individuals (identified as 1 and 5), each cut with three restriction enzymes. Table 1 shows the identity of individual bands on the DGGE filter that was determined both by direct observation of hybridization with specific fragments of each probe and by deduction from the results obtained by hybridization with partially overlapping fragments. Overall, the number and pattern of bands was the same between these two individuals; however, some major differences, such as the *HaeIII* lanes for probes #6 and #7, were observed. Probe #6 hybridized with four major bands for individual 1, but with only three bands for individual 5. Individual 5 lacks the B and D bands and had an extra band just below the A band (Fig. 1C). With probe #7, individual 5 lacked the B band and had an additional band further up the gel (Fig. 1D). *HaeIII* digests of mtDNA from these two individuals were analyzed alongside three other individuals using DGGE. The pattern of individual 5 for the *HaeIII* digest with probes #6 and #7 were different from individuals 1–4 while individuals 1–4 were the same as each other (data not shown). These data are consistent with a missing *HaeIII* site at nt 8251 for individual 5. Since unaffected individuals, like 5, will sometimes demonstrate MBPs, it is impor-

tant to run an appropriate control, such as mtDNA from an unaffected maternal relative, on the same gel as the patient's sample when looking for mutations related to mitochondrial disorders.

Occasionally a diffuse band was seen as "band" G in Fig. 1A. There is an upper band and a lower diffuse smear. Such bands are from fragments which do not have a stable intermediate form with a single denatured melting domain (7); however, their appearance is reproducible and consistent from one individual to the next. Also note that with longer autoradiographic exposures, hybridization to minor bands was observed. However, specific major bands are easily distinguished from the minor bands.

In all, 49 major bands of mtDNA with fragment sizes from 156 to 1166 bp were identified in this study. All 4979 bp of this region are covered by at least one identified fragment and approximately two-thirds (3237 bp) are covered by at least three overlapping identified fragments. Now when a single identified band migrates differently than a control sample in the DGGE system, only the region of mtDNA corresponding to that band needs to be amplified and sequenced to characterize the mutation or polymorphism.

## REFERENCES

1. Aprille, J. R. (1991) *Curr. Opin. Pediatr.* **3**, 1045-1054.
2. Yoon, K. L., Modica-Napolitano, J. S., Ernst, S. G., and Aprille, J. R. (1991) *Anal. Biochem.* **196**, 427-432.
3. Fischer, S. G., and Lerman, L. S. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1579-1583.
4. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981) *Nature* **290**, 457-465.
5. Yoon, K. L., Aprille, J. R., and Ernst, S. G. (1991) *Biochem. Biophys. Res. Commun.* **176**(3), 1112-1115.
6. Yoon, K. L., Ernst, S. G., Rasmussen, C., Dooling, E. C., and Aprille, J. R. (1993) *Pediatr. Res.* **33**(5), 433-440.
7. Myers, R. M., Maniatis, T., and Lerman, L. S. (1987) *Methods Enzymol.* **155**, 501-527.

## A High-Yield Modification of Mutation by Overlap Extension Using Three Primers

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Mutation by overlap extension using four primers is often used for site-directed mutations at sites other than the N- or C-termini (1). The template-independent terminal transferase activity of *Taq* polymerase results in the addition of a single non-template-directed adenine at each 3' end of the duplex PCR<sup>2</sup> fragments (2,3). This reduces the efficiency of mutagenesis. Our modification, which uses three primers in two steps, obviates this and thus results in the correct (mutated) base sequence at the 3' end of the PCR product. Often when using a four-primer method, there is some decrease in efficiency owing to a significant percentage of nonmutated product being produced. We have used the modified procedure in studies on the lens membrane protein MIP26 (4,5), and we find an increase in the efficiency of mutagenesis from 80% for the four-primer method to 100% for our three-primer method. In addition, the yield of mutant PCR product increased from 200 ng per 100  $\mu$ l reaction for the four-primer method to 1  $\mu$ g per 100  $\mu$ l reaction for our three-primer method.

The simple two-step method is outlined in Fig. 1. The site-directed mutagenesis was carried out toward the 3' end of the MIP26 cDNA, to replace asparagine (Asn<sup>244</sup>) by an aspartic acid (Asp) residue to investigate the effect of such substitution on MIP26 structure and calmodulin binding. Primer A (see Fig. 1) was a 35-mer corresponding to the 5' end of MIP26 cDNA. The mutagenic primer B had the sequence

5'-GACTCCAATGGACAG-3'.

This sequence corresponds to nucleotides 724-738 of the cDNA except for nucleotide 730 where A replaced G (underlined in the previously mentioned sequence). This base change converts the codon for Asn at position 244 in the MIP26 into a codon for Asp. Primer C was a 33-mer corresponding to the 3' end of MIP26 cDNA.

The PCR protocol was based on the method of Saiki *et al.* (6). The digested plasmid was incubated with *Taq* polymerase (1 unit per microgram of plasmid) using standard buffer conditions [KCl (50 mM); Tris/HCl, pH 8.3 (10 mM); MgCl<sub>2</sub> (1.5 mM)] in the presence of dTTP (2 mM). Amplification reaction mixes containing primers (500 ng each), template (500 ng), dNTPs, and buffer were heated to 95°C for 5 min prior to the addition of *Taq* polymerase and MgCl<sub>2</sub>. We typically employed 28 cycles of amplification by denaturing DNA at 94°C for 1 min, annealing primers at 50°C for 1.5 min, and extend-primers at 72°C for 1.5 min. After the last cycle of 28 cycles, the samples were left at 72°C for an additional 10 min for a final primer extension.

The mutated fragment of 63 nucleotides (BC in Fig. 1) from the first reaction of overlap extension was recov-

<sup>2</sup> Abbreviation used: PCR, polymerase chain reaction.