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Localization by fluorescence in situ hybridization (FISH) of human mitochondrial polymerase γ (POLG) to human chromosome band 15q24→q26, and of mouse mitochondrial polymerase γ *(Polg)* to mouse chromosome band 7E, with confirmation by direct sequence analysis of bacterial artificial chromosomes (BACs)

S.J. Zullo,[1] L. Butler,[2] R.J. Zahorchak,[2] M. Macville,[3] C. Wilkes,[2] and C.R. Merril[1]

[1] Laboratory of Biochemical Genetics, National Institute of Mental Health, National Institutes of Health, Bethesda, MD; [2] Research Genetics, Inc., Huntsville, AL; and [3] Diagnostic Development Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD (USA)

Abstract. Cloned cDNAs for the human mitochondrial DNA polymerase γ (POLG) were identified by homology with the yeast mitochondrial DNA polymerase catalytic subunit (MIP). Fluorescence in situ hybridization (FISH) of human and mouse bacterial artificial chromosomes (BACs), hybridized by radioactively labeled POLG cDNAs, mapped to human chromosome band 15q24→q26, as well as to mouse chromosome band 7E. Direct sequencing of the BAC DNA without subcloning confirmed the presence of both human POLG and mouse mitochondrial DNA polymerase γ *(Polg)* in the respective BACs.

The maternally inherited human mitochondrial DNA (mtDNA) genome, a closed, circular 16,569 nucleotide pair (np) molecule encoding 13 essential subunits of the mitochondrial respiratory chain, contains very few nucleotides that are not involved in coding either rRNAs, tRNAs, or polypeptides. Thus, deletions in the mtDNA genome can have grave consequences and, indeed, have been implicated in the reduction of metabolic capacity observed with aging (reviewed by Wallace, 1992). Slip-replication has been suggested as one of the possible mechanisms in the formation of mtDNA deletions (reviewed by Wallace, 1992), thus implicating the action of the human mitochondrial DNA polymerase γ (POLG) in the process. Recently, Merril et al. (1996) found a relationship between conditions associated with chronic hypoxia and human mtDNA deletions in specific regions of the brain, confirming a relationship discovered a quarter century ago by Mayer and Legator (1970) in the generation of petite mutants in yeast. Provocatively, mutator alleles of the yeast mtDNA polymerase γ catalytic subunit-encoding gene (MIP-1) have recently been described (Hu et al., 1995) that lead to small ρ^- colonies, a phenotype classically ascribed to deletions of the mitochondrial genome and mirroring the generation of petite mutations found by Mayer and Legator (1970). For these reasons, it would be helpful to clone the human mtDNA POLG to enable in vitro studies of mtDNA synthesis and mutagenesis including mtDNA deletions, as well as the mtDNA depletion syndromes (reviewed by Naviaux, 1997).

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Request reprints from Dr. Steven J. Zullo, Laboratory of Biochemical Genetics, National Institute of Mental Health, National Institutes of Health, Building 10, Room 2D56, Bethesda, MD 20892 (USA); telephone: 301-435-3576; fax: 301-480-9862, e-mail: zullo@helix.nih.gov.

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The catalytic activity of mtDNA polymerases γ across widely diverse species, including yeast (Foury, 1989), *Xenopus laevis* (Insdorf and Bogenhagen, 1989), and human (Gray and Wong, 1992), resides in a 140-kDa subunit. In addition, all known mtDNA polymerases γ are resistant to inhibition by the antibiotic aphidicolin and by dideoxynucleotides, in contrast to eukaryotic nuclear DNA polymerases. Thus, as it is likely that significant sequence identity exists between the polymerases γ of different organisms, it is reasonable to utilize cross-species comparisons to identify the genes and their chromosomal locations.

Materials and methods

The yeast MIP-1 (GenBank ID No. J05117) DNA sequence was PCR amplified (30 cycles of 95 °C for 1 min, 64 °C for 1 min, and 72 °C for 2 min) from yeast genomic DNA in 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 0.01 % gelatin, and 0.5 mM dNTP, in the presence of [α-^{32}P]dA, with the primers SMIP1 (5′-CGACTGTTCACGAGCAGAAAG-3′; nucleotides 1–21 of J05117 sequence) and AMIP1 (5′-AAGACACACTGGGTGGTCC-TA-3′; nucleotides 4694–4674 of J05117 sequence) alongside the identical reaction without labeled dA. Amplification of a 4,694-np MIP1 product was confirmed by electrophoresis of an aliquot of the "cold" reaction, along with corroborative *Bam*HI and *Hin*dIII single digests, on a 1 % agarose gel.

We hybridized the PCR-labeled gene sequence of MIP-1 to a human bacterial artificial chromosome (BAC) library contained on three doubly spotted 22 × 22 cm^2 high-density membranes (Library CITB-978SK-B, Catalog No. 96012; Research Genetics, Inc.). Each membrane contained DNA from 27,648 unique BAC clones, the entire library representing an approximate genome equivalent of 3. Five positive hybridizations were confirmed by the double spotting (Research Genetics Nos. 124D13, 64M23, 27I10, 149K16, and 41M17). A number of weaker hybridizations were not confirmed by the double spotting.

We next identified two I.M.A.G.E. Consortium human cDNA library clones with significant sequence identity to MIP-1 through a BLAST search of the NCBI human expressed sequence tag database (dbEST), and subsequent use of Genetics Computer Group (GCG) sequence analysis software. One human cDNA clone (GenBank ID No. T86774, cDNA clone 115095) possessed 60 % identity over 416 np to the yeast MIP-1 gene sequence, while the second cDNA clone (GenBank ID No. T79099) possessed 66 % identity over 244 np to the yeast MIP-1 gene sequence. In addition, these two cDNA clones are 92 % identical to each other over 302 np, encompassing the same region as their identity to MIP-1. It cannot be determined if there is more than one POLG gene simply from the presence of slightly different cDNAs alone, due to the automated, single-pass sequence reaction used to determine the cDNA sequences. The cDNA clone 115095 (GenBank ID No. T86774) was labeled with phosphorus 32 by PCR (30 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min) with the T7 and T3 vector primers, sequences of which surround the insert. The labeled cDNA was hybridized to the human BAC library high-density membranes, and to the mouse BAC library high-density membranes (Library CITB-CJ7-B, Catalog No. 96022, Research Genetics, Inc.). The same human BAC clones hybridized by the yeast MIP1 probe were hybridized by the cDNA probe. A mouse BAC clone (70N9) was also hybridized by the cDNA probe (MIP-1 was not used to probe the mouse BAC library membranes).

Results and discussion

Four human BAC clones (124D13, 64M23, 27I10, and 149K16) mapped by fluorescence in situ hybridization (FISH) to human chromosome band 15q24→q26 (Fig. 1a). We developed a method to directly sequence the DNA in these BAC clones (see below) which indicated they contain the same sequences; thus, we illustrate here only one. The sequence anal-

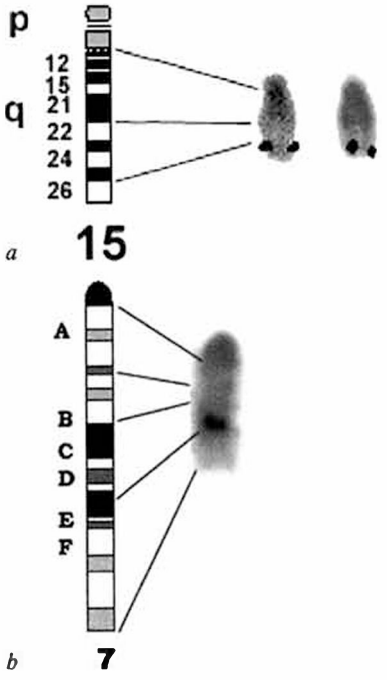

Fig. 1. Comparison of chromosome ideograms with the FISH images resulting from hybridization of human and mouse BAC clones to human and mouse metaphase chromosomes, respectively. (**a**) Four human BAC clones map to human chromosome band 15q24→q26. (**b**) One mouse BAC clone maps to mouse chromosome band 7E. Human metaphase spreads were prepared according to standard protocols, and mouse metaphase spreads were derived from mouse spleen lymphocytes (Boyle et al., 1992). Slides were pretreated for FISH as described by Wiegant et al. (1991). BAC clones were labeled with biotin-16-dUTP by nick translation and hybridized essentially as described by Raap et al. (1990). Biotinylated DNA-DNA hybrids were detected with avidin-FITC. DAPI banding allowed for chromosome identification. Gray-level images were acquired with a Photometrics cooled-CCD camera mounted on a Leica DMRBE epifluorescence microscope equipped with appropriate filter sets and objective lenses.

ysis (Fig. 2) revealed exon, as well as intron, sequences, confirmed by unpublished sequence information kindly provided by W. Copeland (personal communication) and P. Anziano (personal communication), as well as by L. McDaniels and R. Schulz (manuscript submitted for publication and personal communication). One of the human BAC clones, 41M17, mapped by FISH to human chromosome band 6p24; however, sequence analysis did not reveal POLG sequences in the BAC (data not shown). We have not examined this BAC clone any further at this time.

The mouse BAC clone mapped to mouse chromosome band 7E (Fig. 1b). The initial sequence analysis confirmed that the mouse *Polg* gene is present in the identified mouse BAC clone (Fig. 2). We are currently sequencing this mouse BAC clone to fully elucidate the intron/exon arrangement of the mouse *Polg* gene.

Ropp and Copeland (1996) cloned POLG; however, they reported only the cDNA sequence. They also localized POLG to chromosome band 15q24. Additionally, Walker et al. (1997) localized POLG on 15q25 with FISH of a P1 artificial chromo-

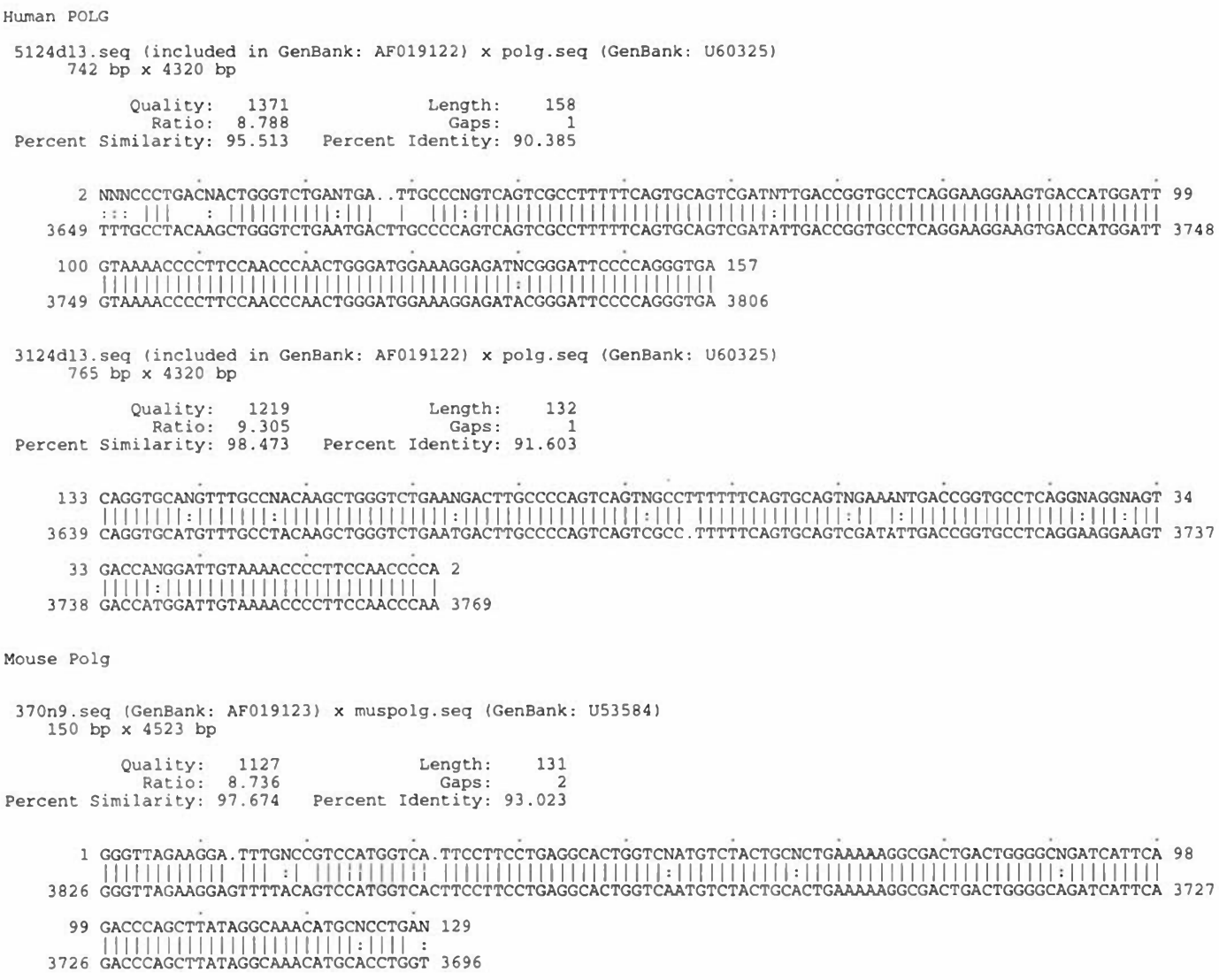

Fig. 2. Identification of human POLG and mouse *Polg* genomic sequences from human and mouse BAC clones, respectively. Best-fit analyses (Wisconsin Package, Version 9, Genetics Computer Group [GCG], Madison, WI) of genomic DNA sequences from human and mouse BAC clones versus the respective cDNAs for human POLG and mouse *Polg*. See text for details.

some (PAC) that had been identified by hybridization with a POLG cDNA clone. In order to generate exon/intron information, as well as to confirm our placements, we utilized the following protocol to sequence directly from the BAC isolates:

Single-colony isolates were inoculated from glycerol stocks into 20-ml cultures. After overnight growth at 37°C, 12.5 ml of each culture was distributed in 2.5-ml aliquots and processed with an AutoGen 740 Automated Nucleic Acid Isolation System (Integrated Separation Systems) using the BAC purification protocol. DNA was resuspended in 50 µl of HPLC-grade water and quantified on a Hoefer TKO100 fluorometer (Hoefer Scientific Instruments). The DNA was either concentrated by vacuum centrifugation or further purified and concentrated by use of Microcon 100 columns (Amicon). In each sequencing reaction, 1 µg of BAC DNA, 6.4 pmol sequencing primer, and 8.0 µl of Terminator Ready Reaction Mix (Perkin-Elmer, Applied Biosystems Division) were used in a total reaction volume of 20 µl. The BAC-end primers used were SP6 (5′-CATACGATTTAGGTGACACTATAG-3′) and T7 (5′-TAATACGACTCACTATAGGGCGA-3′). The internal primers, designed from the cDNA clone 115095 (GenBank ID No. T86774), were POLG-5in (5′-CTTGACCAGGTGCATGTTTGC-3′), POLG-5out (5′-GCCTTGCAGATCACCAACCTC-3′), POLG-3in (5′-GTATCTCCTTTCCCATCCCAG-3′), and POLG-3out (5′-CTGGGATGGGAAAGGAGATAC-3′). The reaction was run on a GeneAmp PCR System 2400 (Perkin-Elmer) using an initial denaturation step of 4 min at 96°C, followed by 40 cycles, each consisting of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, and a final extension holding step for 7 min at 72°C. Unincorporated dye-labeled nucleotides were removed with Centrisep Spin Columns (Princeton Separations), and the products were dried by vacuum centrifugation. Products were resuspended in a formamide/EDTA/blue dextran loading dye, and the entire product was analyzed on an ABI 377 Sequencer using a 5% Longranger gel (FMC Bioproducts).

We were fortunate in that the POLG-5in and POLG-3in primers we designed resided in the same, pentultimate, exon of the genomic POLG sequence, subsequently confirmed by W.C. Coleman (personal communication) and L. McDaniels (manuscript submitted for publication and personal communication)

(Fig. 2). Thus, PCR products and genomic sequence information were generated from all human BACs localizing to chromosome band 15q24→q26, as well as the mouse BAC (Fig. 2). This is the first report of the localization of the mouse *Polg* gene. It is also the first genomic sequence reported of the human POLG and mouse *Polg* genes. Only human POLG cDNA and mouse *Polg* cDNA sequence is currently available in GenBank. All sequences determined in this study have been deposited in GenBank.

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