Nucleotide Sequence Preservation of Human Leukemic Mitochondrial DNA

Raymond J. Monnat, Jr., Clare L. Maxwell, and Lawrence A. Loeb

The Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, University of Washington, Seattle, Washington 98195

ABSTRACT

Nucleotide sequence variation in mitochondrial DNA isolated from human leukemic cells has been analyzed by recombinant DNA techniques. Three hundred eighty-seven independent recombinant DNA clones, each containing one of three defined segments of mitochondrial DNA isolated from the neoplastic cells of four leukemic patients, were analyzed. Partial nucleotide sequence determination of the 387 clones yielded a total of 81.7 kilobases of nucleotide sequence information. The only evidence of within-individual nucleotide sequence divergence consisted of three clones containing deletions of one or two nucleotides in one mitochondrial DNA region. These clones were three of 113 independent clones isolated from a patient with acute lymphocytic leukemia. The low level of nucleotide sequence divergence within the mitochondrial DNA population of neoplastic cells from individual leukemic patients suggests that a mechanism or mechanisms exist that limit the development of nucleotide sequence divergence in mammalian mitochondrial DNA. The results further suggest that this mechanism does not appear to be abrogated by neoplastic transformation in leukemic patients.

INTRODUCTION

Prior to the advent of recombinant DNA techniques, it was not possible to directly identify and characterize mutations occurring in the DNA of normal or neoplastic human cells. Now that individual cellular genes can be isolated, characterized, and reintroduced into mammalian cells, it has become possible to establish causal links between the presence of specific genetic alterations and either resistance to therapy or the development of metastases.

We have applied recombinant DNA techniques to a specific question related to the phenomenon of neoplastic progression. Do mutations accumulate within human neoplastic cell populations? We have examined mtDNA isolated from neoplastic cells of patients with leukemia. We chose to study the mtDNA of human leukemic cells for 3 reasons: (a) mtDNA is well characterized; its nucleotide sequence is known in entirety (3), and a great deal is known about between-individual nucleotide sequence differences; (b) mtDNA can be easily and reproducibly isolated from leukemic cells present in small amounts of peripheral blood; (c) Our previous work (29) demonstrated a low level of nucleotide sequence divergence within the mtDNA population of lymphocytes from individual normal donors. Thus, mtDNA mutations occurring in leukemic cells should be easily detected.

In this paper, we report on the isolation and nucleotide sequence determination of 387 independently isolated recombinant clones containing mtDNA from the neoplastic cells of 4 patients with leukemia.

MATERIALS AND METHODS

Materials. Mononuclear cells were isolated from peripheral blood samples from 4 patients with clinical diagnoses of leukemia. The mononuclear cell fractions were isolated and stored at ~70°C until confirmation of the clinical diagnosis. The cloning vector, M13mp11, and host, Escherichia coli strain JM103, were gifts of Dr. Joachim Messing, University of Minnesota. Protocols for growth of vectors and host strains are given in Ref. 27. Restriction endonucleases, T4 DNA ligase, and M13 sequencing primer were obtained from New England Biolabs or Bethesda Research Laboratories, Inc. Calf intestinal alkaline phosphatase was obtained from Boehringer-Mannheim. E. coli DNA polymerase I large fragment was prepared by digestion of homogeneous E. coli DNA polymerase I (22) with Bacillus subtilis subtilisin, followed by a separation on Sephadex G-100 (23), or obtained from Bethesda Research Laboratories. Ficoll:Hypaque was obtained from Pharmacia Fine Chemicals. Deoxy- and dideoxynucleoside triphosphates and ATP were obtained from Pharmacia P-L Biochemicals. [α-32P]dCTP and [α-32P]dTTP were obtained from New England Nuclear.

Methods. Peripheral blood mononuclear cells were isolated from 4 patients with leukemia by gradient centrifugation in Ficoll:Hypaque (10). The resulting mononuclear cell fractions were washed twice in phosphate-buffered saline, pelleted, and frozen at ~70°C. mtDNA was isolated from the frozen cell pellets by a modification of the "no gradient" technique of Bogenhagen and Clayton (9) [Tapper et al. (38)]. The resulting mtDNA was resuspended in 50 μl of 50 mM Tris (pH 7.5):1 mM EDTA. Prior to cloning, each mtDNA preparation was digested sequentially with the restriction endonucleases SacI and XbaI, extracted with phenol, and precipitated with ethanol. To test for complete digestion, 5 μl (1/10 volume) of each restriction digest containing approximately 10 ng of mtDNA were end-labeled with 2 μCi of [α-32P]dCTP (3000 Ci/mmol) and 0.1 to 0.5 units of T4 DNA ligase. After ligation at 18°C for 12 to 24 h.

The cloning vector, M13mp11, was prepared by cutting the viral replicative form sequentially with the restriction endonucleases SacI and XbaI. The 2 resulting fragments were dephosphorylated with calf intestinal alkaline phosphatase (14), extracted with phenol, and precipitated with ethanol. Ligation reactions were performed in a volume of 30 μl of 50 mM Tris-HCl (pH 7.5):10 mM MgCl2:10 mM dithiothreitol:1 mM ATP containing 100 ng of dephosphorylated M13 DNA, approximately 50 ng of mtDNA, and 0.1 to 0.5 units of T4 DNA ligase. After ligation at 18°C for 24 h, aliquots of the mixture were used to transform the E. coli host strain JM103 (27).

DNA Sequence Analysis. Single-stranded M13 DNA containing mitochondrial inserts was prepared from individual plaque-purified colonies.
by virus precipitation with polyethylene glycol and extraction with phenol (27). DNA sequence determination was by a modification of the dideoxy chain termination method of Sanger et al. (36). Single-stranded recombinant DNA template (0.5 µg) was hybridized with a 2-fold molar excess of a synthetic oligonucleotide sequencing primer (New England Biolabs; 17-mer) in 13 mM Tris-Cl (pH 7.9); 70 mM NaCl; 7.5 mM MgCl₂; annealing was at 55°C for 1 h. Each base-specific chain termination reaction contained in 6 µl of 20 mM Tris-Cl (pH 7.5): 67.5 mM NaCl; 7.5 mM MgCl₂; 10 mM dithiothreitol: 1.67 µM [α-³²P]dTTP (150 Ci/mmol), 0.125 µg of primed viral DNA template, and 0.2 unit of E. coli DNA polymerase I large fragment. The concentrations of limiting deoxynucleoside triphosphates were 4.2 µM for dATP, dCTP, and dGTP and 1.67 µM for dTTP in the respective chain termination reactions. The dideoxy: deoxynucleoside triphosphate ratios used in individual termination reactions were 60:1 for adenosine, 30:1 for cytidine, and 50:1 for thymidine. The concentrations of nonlimiting deoxynucleoside triphosphates were 42 µM in adenosine-, cytidine-, and guanosine-specific base termination reactions and 31 µM in thymidine-specific base termination reactions.

Chain termination reactions were performed at 37°C for 10 min. The sequencing reactions were stopped with 5 µl of 25 mM EDTA (pH 8), vacuum dried, and resuspended in 2 µl of 99% deionized formamide, 10 mM EDTA, 10 mM sodium hydroxide, and 0.3% each of xylene cyanol FF and bromphenol blue. After denaturation for 3 min at 95°C, 1.5-µl aliquots from each reaction were loaded onto a 39- x 33- x 0.035-cm 8% polyacrylamide:7% urea gel. The ratio of acrylamide to bisacrylamide was 19:1; the gel buffer was 135 mM Tris-Cl:45 mM boric acid:2.5 mM EDTA (pH 8.9) (2). After electrophoresis at 1500 V for 2 h, the gels were fixed for 2 min. in 10% acetic acid, transferred onto Whatman No. 3MM chromatography paper, and dried at 80°C for 30 min under vacuum. Autoradiography was performed at room temperature without an enhancer screen for 20 h. All nucleotide sequence differences from the published human mtDNA sequence were verified by repeating nucleotide sequence determination of the template.

RESULTS

mtDNA was isolated from the leukemic peripheral blood mononuclear cells of 4 patients. The 4 leukemic patients were unrelated; 3 of the 4 received no therapy, while one, CML/BC, received chemotherapy a year prior to isolation of the cells. Three different types of leukemia are represented: ALL; CLL; and CML/BC (Table 1).

Purified mtDNA from each patient was cut into 7 fragments by sequential digestion with the restriction endonucleases SacI and XbaI. Of the 4 potential fragments that could be ligated into the cloning vector, M13mp11 cut with SacI and XbaI, 3 (Nos. 4, 5, and 7) were recovered as recombinant molecules (Chart 1). Restriction endonuclease Fragments 4 and 7 span nucleotides 8,287 to 10,256 of the human mitochondrial genome (3). Each of these 2 fragments contains approximately one-half of the gene for CO III. In addition to CO III, Fragment 4 contains genes for ATPase 6, 67S rRNA, and an open reading frame (67L). Restriction endonuclease Fragment 7 contains the gluta myl-tRNA gene and a portion of open reading frame 3, in addition to one-half of the CO III gene. Restriction endonuclease Fragment 5 spans nucleotides 41 to 1,197 of the human mitochondrial genome. Fragment 5 contains the heavy (H) strand origin of mtDNA replication ("D-loop region"), promoters for mtDNA transcription, the phenylalanyl-tRNA gene, and a portion of the 12S rRNA gene (3).

Single-stranded viral recombinant M13mp11 DNA prepared from 387 independently isolated clones containing mtDNA Fragments 4, 5, or 7 was used as a template for nucleotide sequence determination by the dideoxy chain termination method of Sanger et al. (36). The nucleotide sequences of the light (L)-strand of Fragment 4 and of the H-strand of Fragment 7 were determined from the SacI site that divides the CO III gene into halves with a cut between L-strand nucleotides 9647 and 9648. The H-strand sequence of Fragment 5 was determined from the SacI site that cuts between H-strand nucleotides 36 and 37 (Chart 1).

Patient information and a summary of the nucleotide sequence information obtained are given in Table 1. A total of 81.7 kilobases of sequence information, an average of 211 nucleotides per clone, was determined. Single-base substitutions were found approximately once every 230 nucleotides when patient mtDNA sequences were compared with the published human mtDNA sequence. The published sequence was derived largely from mtDNA isolated from a single placenta (3). A small portion of the published sequence (<5%) was derived from HeLa mtDNA or assumed to be identical to bovine mtDNA (3, 4). Single-base substitutions were identified in all patients and in all 3 mtDNA fragments studied (Table 1). Each between-individual substitution in mtDNA Fragment 4, 5, or 7 was found in all independently isolated clones containing that fragment from the same patient. Short deletions of one or 2 nucleotides were identified in 3 of 81 clones containing mtDNA Fragment 7 from one patient (Patient ALL; Table 1). Two of the 3 deletions consisted of a deleted cytidine at L-strand position 9731 or 9732. From which of the 2 positions the cytidine was deleted cannot be determined from the nucleotide sequence of the deletion-containing clones. The third deletion consisted of a deletion of thymidine at L-strand position 9730 and a cytidine from L-strand positions 9727-9 or 9727-12. Again, which cytidine was deleted could not be established unambiguously (Fig. 1). No additional examples of mutations were identified that involved only a portion of independently isolated clones containing the same mtDNA fragment from a single patient.

DISCUSSION

We have used recombinant DNA techniques to identify mtDNA mutations in human leukemic cells. With the exception of 3 clones, we found no evidence of mtDNA nucleotide sequence divergence in 387 independently isolated mtDNA clones isolated from the leukemic cells of 4 patients. The small amount of nucleotide sequence divergence identified in the mtDNAs of 4 leukemic patients has several implications for the biology of human mtDNA and for the pathogenesis of leukemia.

Mitochondrial Nucleotide Sequence Differences between Leukemic Patients. Nucleotide sequence differences in the mtDNAs of unrelated normal humans and other mammals have been documented by restriction endonuclease mapping and by limited DNA-sequencing studies (5, 7, 11–13, 21, 33). In this study, using DNA sequencing, single-base substitutions were identified in each of the 4 patients and in each of the 3 mtDNA fragments studied. Several identical nucleotide substitutions were found in different patients. For example, a substitution of guanosine for adenosine at L-strand position 283 was identified in both of the patients with CML. This substitution occurs in the noncoding "D-loop" region, near the heavy strand origin of mtDNA replication; thus, it does not alter or disrupt a known mitochondrial gene product. This and several other nucleotide
substitutions identified in Patient CLL-1 have been identified in sequence comparisons of the D-loop region of unrelated normal humans (5, 21).

Three additional between-individual differences outside the D-loop region of mtDNA were identified in this study. Substitution of cytidine for thymidine at L-strand positions 9698 and 9725 was found in all clones containing these regions from Patient CML/BC. Both of these base substitutions occur in codon third positions in the CO III gene; neither produces an amino acid substitution.

A third between-individual difference identified in all 4 patients studied was substitution of cytidine for guanosine at L-strand position 9559. This nucleotide substitution was identified by comparing the nucleotide sequence of each patient’s mtDNA Fragment 4 with the published human mtDNA sequence. A substitution of cytidine for guanosine at L-strand position 9559 results in a substitution of proline for arginine at CO III amino acid 118. We identified this base substitution previously in mtDNAs of 5 normal unrelated individuals (29) and have subsequently identified the same base substitution in the mtDNAs of 6 additional unrelated normal or leukemic individuals.4 Thus, it is likely that the guanosine at L-strand position 9559 of the published human mtDNA sequence is an error and/or that the original sequence was from an individual with an uncommon base substitution at this site.

Mitochondrial Sequence Divergence within Individual Leukemic Patients. A major goal of this study was to identify and characterize mtDNA mutations in the neoplastic cells of individual leukemic patients. In a previous study of mtDNAs from lymphocytes of 5 normal unrelated donors, we found that sequence divergence between normal individuals was as great as 0.9%; yet, there was no evidence of within-individual mitochondrial nucleotide sequence divergence at a frequency of greater than 1 in 49,000 nucleotides (0.002%) (29). Against this low background of spontaneous mtDNA mutation, we anticipated that any increase in mutation associated with neoplastic transformation would be easy to detect. We expected nucleotide sequence differences to accumulate in the mtDNA population of an expanding neoplastic cell population?

Many human leukemias appear to be of monoclonal origin, i.e., arise from one or a small number of cells (18). If a neoplasm is of monoclonal origin, the one or small number of neoplastic cells...
from which it originates must double at least 40 times before a sufficient large number of neoplastic cells accumulates to produce the clinical problems by which we recognize leukemia. If the initial neoplastic cell(s) began with a homogeneous mtDNA population, and if the error rate for mtDNA replication is $10^{-4}$ (estimated on the basis of fidelity measurements with mtDNA polymerase) (24), then one nucleotide in 250 should be mutated in each mitochondrial molecule in leukemic cells at the time of diagnosis. These mitochondrial mutations would be in addition to mutations that accumulated during the $\geq 45$ cell population doublings required to convert the zygote into a fully formed organism. Most importantly, a progressive increase in base substitutions would be expected if tumor progression in leukemic cells proceeded by an error-prone DNA replication mechanism that was able to affect mtDNA (25).

In this study, only 3 mitochondrial mutations were identified in 81,700 nucleotides of DNA sequence information obtained from the neoplastic cells of 4 leukemic patients. All 3 of the within-individual differences consisted of deletion of one or 2 nucleotides in the one patient with ALL. These clones were 3 of a total of 81 containing Fragment 7 that were isolated as independent clones from the patient with ALL. The single-base deletions of cytidine in 2 of the clones produce a $-1$ frame shift in the CO III gene and new chain termination codons 24, 48, and 70 downstream from the deletion site. The deletion of a cytidine and thymidine from the L-strand nucleotide region 9727-9732 of the third clone produces a $-2$ frame-shift mutation in the CO III gene and new chain termination codons 4, 26, 70, and 84 downstream from the 2 nucleotide deletion sites.

These within-individual mutations could have originated in at least 3 ways. They could represent (a) preexisting mtDNA variation that was fortuitously amplified with the neoplastic cell population; (b) mutations that originated in the neoplastic cell population; or (c) mutations that arose during mtDNA DNA cloning and/or nucleotide sequence determination. We consider the third possibility unlikely. A previous study of mitochondrial nucleotide sequence divergence in normal individuals, which utilized 78 independently isolated mtDNA clones containing the same region of human mtDNA, failed to reveal either deletion-insertion or nucleotide substitution mutations at positions 9727-9732 (29). Thus, there does not appear to be preferential mutation of these nucleotide positions when human mtDNA is cloned in M13 and the resulting clones used for nucleotide sequence determination by the dideoxy chain termination method.

Concerted Preservation of Human Leukemic mtDNA. Our results strongly suggest that nucleotide sequence divergence is not present in the mtDNA population of neoplastic cells of leukemic patients at a level of greater than one nucleotide substitution per molecule. Two implications of this finding are: (a) that a mechanism (or mechanisms) exist to restrict the development of mitochondrial nucleotide sequence divergence; and (b) that this mechanism does not appear to be abrogated by neoplastic transformation.

With only 3 exceptions, this study revealed an invariant mtDNA population in neoplastic cells of individual leukemic patients. The low level of mitochondrial nucleotide sequence divergence in both normal (29) and neoplastic human cells suggests the existence of a mechanism to limit the accumulation of mutations in the mtDNA population derived from the zygote. How such a mechanism might work is not clear. The most direct mechanism, a passive loss of altered or damaged mtDNA molecules, fails to explain the absence of all nucleotide substitutions, regardless of their coding effects, from human mtDNA. A second mechanism of mitochondrial nucleotide sequence preservation could be more accurate mtDNA replication or mtDNA repair. For example, a hundredfold increase in the accuracy of mtDNA replication, predicted from measurements of the fidelity of the suspected mtDNA polymerase, $\gamma$, in vitro (24) would be sufficient to limit the development of mitochondrial nucleotide sequence divergence during development. However, neither more faithful mtDNA replication nor mtDNA repair appears adequate to limit mutation accumulation during mtDNA turnover in normal somatic cells following completion of development (35) or in an expanding neoplastic cell population.

A third possible mechanism that could explain the absence of nucleotide substitutions in the mtDNA population of an individual is the repeated correction of all mtDNA molecules in a cell against a master copy. This mechanism in its simplest form is unlikely to be correct, as genetically distinct mtDNA molecules appear to be able to persist in single human cells (39).

In this study, the nucleotide sequence of mtDNA isolated from the neoplastic cells of individual leukemic patients was found to be highly conserved. Thus, critical tests of a somatic mutational origin of tumor cell heterogeneity and tumor progression (19, 20, 25, 32, 34, 40) must focus on defined nuclear genes. The methods and approach developed in the course of this investigation can be used to identify and characterize nucleotide sequence divergence in specific nuclear genes from individual tumor cell populations. Two families of nuclear genes that will be particularly important to analyze in this manner are cellular...
protooncogenes and those genes whose products play essential roles in cell growth and division (8, 28).

ACKNOWLEDGMENTS

We thank Dr. Bernard Poiesz and Dr. Marshall Kadin for providing leukemic cells and R. Marlene Koplitz for help with DNA sequence determinations.

REFERENCES

Fig. 1. Nucleotide deletions identified in mtDNA Fragment 7 of Patient ALL. The published L-strand sequence of nucleotide positions 9721 to 9738 of human mtDNA is given (left). Seventy-eight of 81 clones containing Fragment 7 from Patient ALL had a nucleotide sequence identical to that of the published sequence. The nucleotide sequencing gel autoradiogram of one of these 78 clones is shown (Lanes 1 to 4). Two of 81 clones contained a deleted cytidine at position 9731 or 9732. The nucleotide sequencing gel autoradiogram of one of these 2 clones is shown (Lanes 5 to 8). One of 81 clones contained a deletion of the thymidine at position 9730 and of a cytidine from positions 9727 to 9729 or 9731 to 9732. The nucleotide sequencing gel autoradiogram of this one double-deletion clone is shown (Lanes 9 to 12). *, it is not possible to assign the deleted cytidines unambiguously to positions 9727 to 9729 or 9731 to 9732.
Nucleotide Sequence Preservation of Human Leukemic Mitochondrial DNA

Raymond J. Monnat, Jr., Clare L. Maxwell and Lawrence A. Loeb


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/45/4/1809

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.