Identification and Characterization of Mitochondrial Genome Concatemers in AIDS-Associated Lymphomas and Lymphoma Cell Lines

Felipe Bedoya
University of South Florida

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Identification and Characterization of Mitochondrial Genome Concatemers in AIDS-Associated Lymphomas and Lymphoma Cell Lines

by

Felipe Bedoya

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Date of Approval:
June 5, 2009

Keywords: mitochondrial DNA, AIDS-related lymphomas, oncogenic transformation, leukemogenesis, mitochondria.

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I would like to dedicate this work to all the people who have led me to and through this wonderful and exciting path of scientific research. I want to give special thanks to my mentor Peter Medveczky who has guided me throughout this project, and from whom I have learned not only some of the fundamental techniques in molecular biology, but also the skills necessary for research conduct, scientific writing and data analysis. I want to thank also Maria Medveczky for always being helpful and for sharing with me her technical knowledge and expertise. Working in their laboratory and sharing with them both (and their laboratory members) these past three years has been one of the most enjoyable experiences I have ever had. I am very grateful with my committee members Drs. Burt Anderson, George Blanck and Edward Seto for their advice and constructive criticism of my project. Finally, I want to express my gratitude to all the professors, students and staff whom I have interacted with during my graduate studies. They have been essential in my getting to the point where I am now.
# Table of Contents

List of Tables .................................................................................................................. v
List of Figures ..................................................................................................................... vi
List of Abbreviations .......................................................................................................... viii
Abstract .............................................................................................................................. x

Introduction ........................................................................................................................ 1

- AIDS-Associated Lymphomas ......................................................................................... 1
- Mitochondria ..................................................................................................................... 4
- The Mitochondrial Genome ............................................................................................. 6
- Particularities of the Mitochondrial Genome ................................................................. 9
  - Polyploidy ...................................................................................................................... 9
  - Non-Mendelian (Maternal) Inheritance ........................................................................ 9
  - Evolution Rate .............................................................................................................. 10
  - Divergent Genetic Code ............................................................................................... 10
  - Gene Organization ....................................................................................................... 11
- Mitochondrial DNA Transcription .................................................................................. 14
- Mitochondrial DNA Replication .................................................................................... 16
List of Tables

Table 1. Classification of AIDS-Associated Lymphomas According to WHO ................................................................. 3

Table 2. Oligonucleotides Used for PCR Amplification and Sequencing of EL CccDNA and MtDNA of Normal T-Cells ........................................................................................................ 34

Table 3. DNA Sequencing Results of EL CccDNA Cloning .......................................................................................... 49
List of Figures

Figure 1. The Mitochondrion ................................................................. 5
Figure 2. The Human Mitochondrial Genome ..................................... 8
Figure 3. Schematic Representation of the Mammalian D-Loop .......... 13
Figure 4. Strand-Displacement Model of Mammalian MtDNA Replication .......................................................... 18
Figure 5. Strand-Coupled Model of Mammalian MtDNA Replication .......................................................... 20
Figure 6. The Gardella Gel Technique and Visualization of Large CccDNA in an AIDS-Associated Lymphoma Sample (EL) .......................................................... 42
Figure 7. Southern Blotting Analysis of EL CccDNA Using Probes Specific for Both EBV and KSHV ...................... 44
Figure 8. EL Cells are CD4+ T-Lymphocytes .......................................... 45
Figure 9. Gel Electrophoresis Analysis of CsCl-EtBr Gradient Fractions of Total EL DNA ........................................ 46
Figure 10. Schematic Representation of the Method Performed for Isolation and Analysis of EL CccDNA ................. 48
Figure 11. Gel Electrophoresis and Southern Blotting of Purified CccDNA from EL and Normal T-Cells Using MtDNA-Derived Probes ........................................ 50
Figure 12. Electron Microscopy of EL CccDNA ..................................... 52
Figure 13. Complete and Partial BamHI-Restriction Digestion of EL CccDNA ................................................................. 53

Figure 14. Multiple Sequence Alignment Analysis of Conserved Sequence Box II (CSBII) Regions from EL and Normal MtDNA ............................................................. 55

Figure 15. Flow Cytometric Analysis of Mitochondrial Physiological and Structural Properties ................................................................. 57

Figure 16. Mitochondrial Genome Concatemers are Present in Primary AIDS-Associated Lymphomas and Lymphoma Cell Lines .................................................................. 58

Figure 17. Gel Electrophoresis and Densitometric Analyses of CccDNA from Human Lymphoma Cell Lines .................................................................................. 60

Figure 18. Southern Blotting Assay of Gardella Gel of Herpesvirus-saimiri-Immortalized T-Cells Reveals the Presence of Mitochondrial Genome Concatemers ............ 62

Figure 19. Densitometric Analysis of Mitochondrial DNA from Herpesvirus-saimiri-Immortalized and Normal T-Cells ........................................................................ 63

Figure 20. Schematic Model of MtDNA Configurations Identified in AIDS-Associated Lymphomas and Lymphoma Cell Lines .............................................................. 65

Figure 21. DNApol-γ (Subunit A and B) mRNA Levels in Several Human Tissues and Human Cell Lines (GNF Human Gene Atlas) ........................................................................ 72

Figure 22. MtssB and Twinkle mRNA Levels in Several Human Tissues and Human Cell Lines (GNF Human Gene Atlas) ................................................................. 73

Figure 23. Inhibition of DNA Polymerase Gamma by NRTIs (AZT) ...................................................................................... 76

Figure 24. Schematic Diagram of Proposed Mechanism of Induction of Oncogenic Transformation by Formation and Maintenance of Mitochondrial Genome Concatemers ................................................................. 84
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δψm</td>
<td>Mitochondrial Transmembrane Potential</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>AZT</td>
<td>Azidothymidine</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CCC</td>
<td>Covalently Closed Circular</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSBII</td>
<td>Conserved Sequence Box II</td>
</tr>
<tr>
<td>CsCl</td>
<td>Cesium Chloride</td>
</tr>
<tr>
<td>DHE</td>
<td>Dihydroethidium</td>
</tr>
<tr>
<td>DLBCL</td>
<td>Diffuse Large B-Cell Lymphoma</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNAPOL-γ</td>
<td>DNA Polymerase Gamma</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HL</td>
<td>Hodgkin’s Lymphoma</td>
</tr>
<tr>
<td>HVS</td>
<td>Herpesvirus Saimiri</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s Sarcoma-Associated Herpesvirus</td>
</tr>
<tr>
<td>MNGIE</td>
<td>Mitochondrial Neurogastrointestinal Encephalomyopathy</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>MtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NAO</td>
<td>10-N-Nonyl Acridine Orange</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>NHL</td>
<td>Non-Hodgkin’s Lymphoma</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative Phosphorylation</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PEL</td>
<td>Primary Effusion Lymphoma</td>
</tr>
<tr>
<td>PEO</td>
<td>Progressive External Ophthalmoplegia</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PTLD</td>
<td>Post-Transplant Lymphoproliferative Disorder</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SANDO</td>
<td>Sensory Ataxia, Neuropathy, Dysarthria and Ophthalmoplegia</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethyl Rhodamine Methyl Ester</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Identification and Characterization of Mitochondrial Genome Concatemers in AIDS-Associated Lymphomas and Lymphoma Cell Lines

Felipe Bedoya

ABSTRACT

Despite recent advances in the understanding of the molecular bases of hematological malignancies, the specific mechanisms on how they originate and why some subtypes are more prevalent than others still remain to be elucidated. These two important aspects have been even more difficult to analyze when dealing with individuals under immune suppression because other factors must be considered. Questions still remain as to why individuals with AIDS tend to develop lymphoproliferative disorders differently from those observed in individuals under iatrogenic immunosuppressive therapy. Most of lymphomas occurring in transplant recipients are B-cell neoplasias typically associated with Epstein-Barr virus (EBV) infection. In contrast, only about 50% of lymphomas of patients with AIDS are associated with lymphotrophic herpesviruses such as EBV and Kaposi’s sarcoma-associated herpesvirus (KSHV). No known infectious agent has been detected in the remaining 50% of
AIDS-associated lymphomas, suggesting the involvement of novel viruses or unique molecular mechanisms. Since most oncogenic viruses persist as episomal circular viral genomes in the nuclei of tumor cells, we developed a method to visualize and identify covalently closed circular DNA (cccDNA) in lymphoma samples. Although this study revealed no novel viruses, we identified concatemers of the mitochondrial genome in all lymphoma samples tested. We further studied in detail one AIDS-associated lymphoma (denominated EL) whose mitochondrial DNA primarily consisted of tandem head-to-tail genome duplications. Insertion of cytosine residues was noted in the EL mitochondrial genome sequence near the origin of replication. EL cells responded weakly to Fas-apoptotic stimulus, displayed reduced mitochondrial activity and mass, and produced higher levels of reactive oxygen species (ROS) than control cells. Screening of several other AIDS-associated lymphomas and established lymphoma cell lines revealed a different kind of mitochondrial genome concatemers consisting of interlinks of DNA monomer molecules. Concatemers were not detected in normal T-lymphocytes suggesting an association with neoplastic transformation. This dissertation describes the two distinct types of mitochondrial genome concatemers identified in transformed lymphoid cells and presents a detailed analysis of their structure and implications in cellular homeostasis.
Introduction

AIDS-associated lymphomas

After the acknowledgment of HIV as the causative agent of AIDS, it was soon recognized that the incidence of lymphoma in HIV-infected patients greatly exceeded that in the general population. The relative risk of non-Hodgkin’s lymphoma (NHL) is increased 60-200 fold in individuals with AIDS (12, 58, 147). The increased risk of lymphoma has been attributed to multiple factors, including the capability of the retrovirus itself to induce transformation (86, 101), the cytokine dysregulation and immunosuppression associated with the disease (28, 82), and the opportunistic infections caused by lymphotrophic \( \gamma \)-herpesviruses such as Epstein–Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV) (16, 89, 136, 209). Several AIDS-associated lymphoma subtypes have been classified according to the heterogeneous morphology observed in patients. The WHO categorizes the HIV-associated lymphomas into (1) those also occurring in immunocompetent patients, (2) those occurring more specifically in HIV-positive patients, and (3) those also
occurring in patients with other forms of immunosuppression (Table 1).
Notably, the majority of these lymphomas are aggressive B-cell neoplasms that also occur in immunocompetent patients (17, 148).

Highly active antiretroviral therapy (HAART) has been reported to both increase and decrease the incidence of different lymphoma subtypes. For example, immunoblastic diffuse large B-cell lymphoma (DLBCL) was reduced from 38% in the pre-HAART era to 19% in the post-HAART era. Also, primary CNS lymphoma incidence decreased from 28% to 17%. However, this trend has not been observed in all AIDS-related lymphomas, centroblastic DLBCL increased from 21% to 44% of cases, and Burkitt’s lymphoma increased from 4% to 9% (43, 135). Several studies have shown that the level of immunosuppression is also associated to the lymphoma subtype that develops in AIDS-patients. For instance, Burkitt’s and centroblastic DLBCL are commonly diagnosed in partially immunodeficient individuals, whereas primary effusion lymphoma (PEL) and immunoblastic DLBCL are most often present in AIDS-patients suffering from severe immunodeficiency (16, 63).
| 1) Lymphoma also occurring in immunocompromised patients | • Burkitt's and Burkitt's-like lymphoma  
• Diffuse large B-cell lymphoma (DLBCL)  
  • Centroblastic  
  • Immunoblastic (including primary CNS lymphoma)  
• Extranodal marginal zone lymphoma of MALT type  
• Peripheral T-cell lymphoma  
• Classical Hodgkin's lymphoma (HL) |
|----------------------------------------------------------|---------------------------------------------------------------|
| 2) Lymphoma occurring more specifically in HIV-positive patients | • Primary effusion lymphoma (PEL)  
• Plasmablastic lymphoma of the oral cavity type |
<table>
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<tr>
<td>3) Lymphoma also occurring in other immunodeficiency states</td>
<td>• Polymorphic B-cell lymphoma (PTLD-like)</td>
</tr>
</tbody>
</table>

Table 1. Classification of AIDS-associated lymphomas according to WHO.
Mitochondria

Mitochondria are semi-autonomous subcellular organelles essential for normal cell physiology and cellular homeostasis (196). Mitochondria are the only organelles that contain their own genome and protein synthesis machinery – possibly due to their prokaryotic endosymbiont ancestry (98, 196). They are motile and undergo frequent changes in morphology and number through fusion and fission events. Even in the same cell, mitochondria can adopt a different range of morphologies (23, 42). It is in the mitochondria where many important metabolic reactions take place such as the urea cycle, lipid metabolism, steroid hormone and porphyrin synthesis and interconversion of amino acids (164, 195). Moreover, mitochondria play a central role in complex physiological processes including cellular proliferation, differentiation, apoptosis (60, 139, 150), and in cellular processes like glucose sensing, insulin regulation (15), cellular Ca\(^{2+}\) and ROS homeostasis (123, 176). Hence, it is not surprising that mitochondrial dysfunction has been associated with degenerative diseases, aging and cancer (30, 61, 155, 198).
Figure 1. The mitochondrion.

Diagram showing energy (ATP) production from oxidative phosphorylation (OXPHOS), reactive oxygen species (ROS) production, and initiation of apoptosis through the mitochondrial permeability transition pore (mtPTP). The OXPHOS complexes, designated I to V, are complex I (NADH:ubiquinone oxidoreductase), complex II (succinate:ubiquinone oxidoreductase), complex III (ubiquinol:cytochrome c oxidoreductase), complex IV (cytochrome c oxidase), and complex V (H+-translocating ATP synthase). Pyruvate from glucose enters the mitochondria via pyruvate dehydrogenase (PDH), generating acetyl-CoA, which enters the TCA cycle by combining with oxaloacetate. Lactate dehydrogenase (LDH) converts excess pyruvate to lactate. The voltage-dependent anion channel (VDAC) allows passage of small molecules through the inner and outer membrane. VDAC together with ANT and Bax are thought to come together at the mitochondrial inner and outer membrane contact points to create the mitochondrial permeability transition pore (mtPTP). The mtPTP interacts with the pro-apoptotic Bax, anti-apoptotic Bcl2 and the benzodiazepine receptor (BD). The opening of the mtPTP is associated with the release of cytochrome c (CytC) and several pro-apoptotic factors that interact with and activate cytosolic Apaf-1, which then binds to and activates procaspase-9. The activated caspase-9 then initiates the proteolytic degradation of cellular proteins ending in apoptosis.
The mitochondrial genome

The mammalian mitochondrial genome is a covalently closed circular (ccc) duplex DNA (mtDNA) with species-specific contour length of about 5μm and nucleotide composition range around 16 Kbp (41, 98). In humans, the mtDNA encodes 13 genes involved in the respiratory chain: 7 genes for complex I (NADH-dehydrogenase-ubiquinone reductase): NADH dehydrogenase subunit 1 (ND1), ND2, ND3, ND4, ND4L, ND5 and ND6; 1 gene for complex III (ubiquinol-cytochrome c reductase): cytochrome b; 3 genes for complex IV (cytochrome c oxidase): Cytochrome c oxidase subunit 1 (COX1), COX2 and COX3, and 2 genes for complex V (ATP synthetase): ATPase6 and Atpase8 (1).

The mtDNA also encodes 22 transfer RNAs, and two ribosomal RNAs known as 12S and 16S rRNAs, that are exclusive for the translation of the 13 mtDNA protein-coding genes. All of the peptides and mature transcripts derived from the mtDNA are necessary for the proper function of the oxidative phosphorylation (OXPHOS) machinery, which is the main biochemical pathway for the synthesis of ATP(1, 197).

In average, 200-10,000 copies of mtDNA are present in a single cell depending on cell type and cellular energy demand. At birth, most (if not all) of
the mtDNA copies are identical (homoplasmic) owing to their maternal origin (65, 108). However, degenerative cellular processes (i.e. ageing, disease, etc.) have been implicated in the causality of several mitochondrial and mtDNA abnormalities, and vice versa (42, 184, 198). Unlike nuclear DNA, the mtDNA is constantly turned over and replicated during the whole cell cycle in normal cells (77, 80).

To date, a large number and ample variety of mtDNA mutations have been described, and over 200 human disorders are associated with specific point and frame-shift mutations, fusions, partial deletions, or depletion of mtDNA. For instance, several mtDNA mutations have been reported recently in human colorectal cancers, and bladder, head, neck and lung tumors (14, 27, 30, 73, 95, 184).

Clinically defined syndromes such as MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes), MERRF (myoclonus epilepsy and ragged-red fibres), and Kearns-Sayre syndrome (retinitis pigmentosa, progressive external ophthalmoplegia, ataxia and heart conduction defects) have all been directly associated to atypical mtDNA forms (94, 172, 214).
Figure 2. The human mitochondrial genome.

Human mtDNA-encoded genes: NADH dehydrogenase subunits of complex I (ND1-ND6 and ND4L), cytochrome b (Cytb) of complex III, cytochrome c oxidase subunits of complex IV (COX1-3), ATP synthase subunits 6 and 8 of complex V, rRNAs (12S and 16S), and tRNAs (indicated by the one-letter amino acid symbol). Also showing control region (D-loop) and arrows indicating the promoters for transcription of the heavy (HSP) and light (LSP) strand of mtDNA and the origins of leading (OH) and lagging (OL) strand replication. Modified from reference 31.
Particularities of the mitochondrial DNA

In comparison to the nuclear genome, several characteristics have been exclusively attributed to the mtDNA:

1) Polyploidy: Most mammalian cells contain hundreds of mitochondria and each mitochondrion contains several copies (2-10) of the mtDNA (131). It is believed that a single individual has identical copies of the mtDNA; however, mutations can arise and co-exist with wild-type mtDNA, a phenomenon known as heteroplasmy. Different levels of heteroplasmy have been reported in different cell types and tissues due to random segregation of mitochondria (and mtDNA) at cell division (174). In order to produce a particular phenotype (e.g. disease), the level of heteroplasmy has to reach a certain percentage known as the “threshold effect”, usually around 60-80% of total mtDNA (44, 152).

2) Non-mendelian (maternal) inheritance: A mammalian oocyte contains around 100,000 copies of the mitochondrial genome – a large amount of mtDNA in comparison with the mere 100 copies carried in a single sperm cell (most of them surrounding the sperm tail to provide ATP for motility) (175). Therefore, during fertilization, the sperm head (containing the nucleus) is fused
to the egg, and basically no mitochondria are transferred in the process; the few mitochondria that could enter in the newly formed zygote are actively eliminated by a ubiquitin-dependent mechanism (180, 208). Recently, new evidence showing that paternal mtDNA can be present in the offspring has been reported, although the mechanism remains unknown (93, 167).

3) Evolution rate: There are no histones in the mitochondrion; therefore, the mtDNA is less protected than the nuclear genome (168). Moreover, the mtDNA is physically associated with the inner mitochondrial membrane where damaging ROS are present, and less-efficient DNA repair mechanisms have been identified in mitochondria (168, 177, 206). Together, these reasons explain why the mtDNA has higher mutation rates than the nuclear DNA, and provide a hint on how somatic accumulation of mtDNA mutations can play a role in human aging and disease. On the other hand, single nucleotide polymorphisms (SNPs) in the mtDNA have been mapped and have served as a tool for human population genetics and evolutionary studies (62, 201).

4) Divergent genetic code: Mitochondrial genes are translated by a mtDNA-restricted translation machinery that utilizes a genetic code distinct from
the universal genetic code. For example, in mammals, UGA rather than a termination codon codes for tryptophan, and AGA and AGG are termination codons instead of encoding arginine (7).

5) Gene organization: One of the main characteristics of mammalian mtDNA is its compact gene organization. All the genes are next to each other or separated by a few nucleotides with no introns and no 5’ or 3’ non-coding regions (1). In human mtDNA, overlapping occurs between genes ATPase 6 and ATPase 8 (46 nt in common), and between ND4 and ND4L (7 nt in common) (6) (Figure 2). And for several genes, the termination codon is added by poly-adenylation of the mRNA, since it is not encoded in the mtDNA (32). The tRNAs are interspersed between genes and play an important role in RNA processing (140). Post-transcriptional excision of tRNAs from polycistronic messages is mediated by the mitochondrial RNase P enzyme (encoded in the nucleus). This process of excision is necessary for the expression of the mitochondrial genomic information (45, 189).

The overall genetic content and organization of vertebrate mitochondrial genomes is highly conserved; however, the mtDNA sequence and the position of regulatory elements for replication and transcription vary considerably even within a single species (168). Two major non-coding regions are present in the
mammalian mtDNA: the D-loop and the origin of replication for the light (L) strand (O_L). The D-loop (~1kb) contains the origin of replication for the heavy or H-strand (according to its higher guanine content) and the promoters for both H- and L-strand transcription (168, 183). Also, within the D-loop highly conserved regions are found throughout several metazoan mtDNAs. They are known as the conserved sequence boxes (CSB I-III) and the termination-associated sequences (TASs) (47, 168). The O_L is a 30 nucleotide-long segment at about two-thirds of the mtDNA (from O_H) that is located inside a tRNA cluster known as the “WANCY” region (from the amino acids single-letter nomenclature) (Figure 3) (34, 47).
Figure 3. Schematic representation of the mammalian D-loop.

Diagram showing the non-coding regulatory D-loop and transcription termination regions. Also, the main factors and cis-elements involved in mtDNA transcription and replication. DNA polymerase-γ (DNAPol-γ), exclusive for mtDNA replication. The mitochondrial RNA polymerase (POLRMT) binds to mitochondrial transcription factors (MTFs) bound to regions upstream of promoters for light-strand (LSP) and heavy-strand (HSP1 and HSP2) transcription. RNase P is a ribonucleoprotein that processes the transcripts from LSP to generate the primers for heavy-strand replication from its origin (OH). Conserved sequence boxes (CSB I-III) and termination-associated sequences (TAS) important for transcription and replication of the heavy-strand.
Mitochondrial DNA transcription

In human mtDNA, independent promoter regions for transcription initiation of each strand have been described (33). The light-strand (lower guanine content) encompasses one promoter region known as \textit{LSP}, and the heavy-strand contains two, \textit{HSP1} and \textit{HSP2} (32, 33). Polycistronic precursor RNAs (some of them nearly full-genome long) are transcribed from both \textit{LSP} and \textit{HSPs} by the mitochondrial DNA-dependent RNA polymerase (\textit{POLRMT}) (13). Mature mRNAs and rRNAs are then separated by excision of the tRNAs (flanking all the protein-coding genes). Processing of the mitochondrial RNA precursors is carried out by RNase P. This mechanism of RNA processing has been described as a “tRNA punctuation model” (13, 140, 189).

Since POLRMT cannot directly bind DNA and initiate transcription on its own, it requires assistance from mitochondrial transcription factors (mtTFA and mtTFB) that associate with specific regions upstream of the mtDNA promoters (5, 46). Although ubiquitous expression, there is substantial evidence indicating that the mammalian mitochondrial transcription machinery is differentially regulated throughout tissues (48, 161). Notably, all the proteins involved in mtDNA transcription and production of mature mtRNAs are encoded in the nuclear genome (156).
Since mitochondrial gene expression is essential for OXPHOS, an appealing idea, that has not been fully addressed so far, is how mtDNA transcription is coordinated with cellular energy demand. Different cell and tissue types have energetic demands that vary according to their physiological state, which in turn require different rates of mitochondrial biogenesis (165, 194). The mechanisms that govern mitochondrial biogenesis are poorly understood; however, some factors and pathways have been characterized for specific situations. During cellular proliferation, for example, upregulation of mitochondrial and nuclear genes necessary for the proper assembly and function of the OXPHOS system have been documented (13, 163). This process requires the participation of protein activators such as nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2), CREB, Sp-1 and others (160, 162). Equally necessary, are coactivators that interact with nuclear DNA-binding proteins such as PGC-1α and PRC, in order to regulate gene transcription in response to environmental and proliferative signals (2, 83, 160).

MtDNA-transcription has been shown to be also regulated by intracellular ATP levels (66, 128, 192). Shuey et al. demonstrated that a “sensing mechanism” of the amounts of ATP is responsible for the differential expression of mitochondrial transcripts, since ATP is required 15- to 20-fold more over the other NTPs for transcription (170). Furthermore, recent evidence
shows that differential transcription from LSP versus HSP1 occurs depending on ATP availability, resulting in altered mRNA and rRNA profiles (51, 134).

Mitochondrial DNA replication

Mitochondria possess a unique protein system devoted exclusively to mtDNA replication. Most of the components of this repertoire have been characterized; however, some elements still remain to be identified since there seems to be no apparent mitochondrial counterpart for the corresponding nuclear enzymatic activity (e.g. primase for mtDNA light-chain synthesis) (46). An important finding in the study of mtDNA is that replication is totally dependent on transcription. This is due to the fact that replication of the heavy strand at the origin (O_H) is primed by a short RNA transcribed from the LSP region (24, 25). This phenomenon has been demonstrated by several groups. Chang D. et al. showed that using mitochondrial transcription assays in vitro some of the nascent transcripts remain bound to the mtDNA at the O_H (26). And Kang et al. detected in vivo RNA-primed DNA strands using the polymerase chain reaction analysis (81).
Although the exact mechanism is still under intense debate, two major models of mtDNA replication have been proposed (31). The first one (in terms of years from publication) is the strand-displacement replication, and the other is the strand-coupled replication. The strand-displacement mechanism was initially proposed in 1982 by Clayton et al. from direct observation of purified mtDNA by electron microscopy (34). This mode of mtDNA replication resembles to that of bacterial plasmids (e.g. ColE1), but is different from that of chromosomal DNA replication of both eukaryotes and eubacteria. It posits that replication of the heavy- (leading) strand initiates at O_H and proceeds unidirectionally until it reaches two-thirds of the genome, where the origin of the light- (lagging) strand (O_L) is exposed on the displaced parental strand, initiating replication in the opposite direction (Figure 4). This model, also called the asynchronous, strand-asymmetric replication model, introduces the concept of a displacement loop (D-loop) observed at high frequency in the purified mtDNA analyzed. The D-loop was described as a third short heavy DNA strand associated to the parental mtDNA of about 1Kb of length and presumably formed by 5’ to 3’ mtDNA synthesis arrest at the termination-associated sequences (TASs) (168). D-loops of different size have also been detected in mtDNA from other species (100, 159).
Figure 4. Strand-displacement model of mammalian mtDNA replication.

Closed circular mtDNA enters replication by formation of D-loop. Migration of the heavy-strand continues unidirectionally displacing the parental strand until it reaches two-thirds of the mtDNA, where the origin for light-strand ($O_L$) is exposed initiating replication in the opposite direction.
The strand-coupled mechanism of replication was proposed more recently by Jacobs and colleagues (70). It is based on the detection of replicative mtDNA intermediates in 2D-gels. This technique separates DNA fragments based on size and shape. Therefore, replication intermediates are expected to resolve in predictable ways. In case both DNA strands replicate simultaneously they tend to form a replication fork, which can be detected as a “Y arc” in 2D-gels. Such Y arcs were unambiguously identified when analyzing mtDNA from human placenta and mouse liver, underscoring the existence of a strand-synchronous replication mechanism; however, single-stranded intermediates were also detected meaning that a combination, rather than a mutually exclusive mode, of both mechanisms might be present in mammalian mitochondria (149) (Figure 5).

An interesting observation perhaps is that not all genes required for mammalian mtDNA replication have evolved from a prokaryotic ancestor. For example, POLRMT, the catalytic subunit of mtDNA polymerase (POLγA), and the mtDNA helicase (TWINKLE) are similar to proteins encoded by bacteriophages of the T-odd lineage, which include bacteriophages T3 and T7. Therefore, it has been proposed that some genes necessary for replication and expression of the mtDNA were acquired from a T-odd phage ancestor early in the evolution of eukaryotic organisms, possibly during the time when mitochondrial endosymbiosis occurred (21, 171).
Figure 5. Strand-coupled model of mammalian mtDNA replication.

Closed circular mtDNA enters replication by formation of a D-loop that facilitates bidirectional polymerization of leading and lagging strands from origin $O_H$ (it could also start from $O_L$) at the same time (synchronous). Migration could proceed around the genome with lagging-strand replication being the result of multiple short-fragment ('Okazaki') synthesis.
Oxidative phosphorylation

Mitochondria generate energy by oxidizing metabolites derived from our dietary carbohydrates (TCA cycle) and fats (β-oxidation) to generate heat and ATP (Figure 1) (198). Embedded in the inner mitochondrial membrane, there is a system of electron carrier complexes, also known as the electron transport chain (ETC). The main function of the ETC is to transfer electrons by a multi-step redox process from TCA cycle products (NADH and FADH$_2$) to oxygen, which is reduced to water (36, 49, 198). Electrons are initially donated from NADH and FADH$_2$ to complex I (NADH dehydrogenase) or to complex II (succinate dehydrogenase), respectively. Ubiquinone (also coenzyme Q or CoQ) carries these electrons to give ubisemiquinone (CoQH•) and then ubiquinol (CoQH$_2$). Ubiquinol transfers its electrons to complex III (ubiquinol:cytochrome c oxidoreductase), which transfers them to cytochrome c, an electron carrier protein located within the intermembrane space. Then, cytochrome c carries the electrons to complex IV (cytochrome c oxidase or COX) and finally to ½O$_2$ to give H$_2$O. In order to transfer electrons, each ETC complex contains multiple electron carriers. Complexes I, II, and III encompass several iron-sulfur (Fe-S) centers, whereas complexes III and IV contain the b + c1 and a + a3 cytochromes, respectively (169, 199, 200, 202).
The energy released by the flow of electrons through the ETC is used to pump protons out of the mitochondrial inner membrane through complexes I, III, and IV. The efflux of protons creates a pH gradient and an electrochemical gradient across the mitochondrial inner membrane. This proton gradient is harnessed by complex V ($F_0F_1$ ATP synthase) for ATP production. As protons flow back into the mitochondrial matrix through a proton channel in complex V, ADP and inorganic phosphate ($P_i$) are bound, condensed, and released in the matrix as ATP. Then, matrix ATP is exchanged for cytosolic ADP through the adenine nucleotide translocator (ANT) (Figure 1). As protons flow through the ATP synthase proton channel, the proton gradient is depolarized. Since the ETC is coupled to ATP synthesis, this process is known as oxidative phosphorylation (OXPHOS). The coupling efficiency of the OXPHOS system determines the efficiency of producing ATP from dietary calories (36, 172, 198, 202).

Mitochondria and cancer

Cancer cells utilize glycolytic pathways for energy generation while upregulating their aerobic respiratory activity. This metabolic change discovered by Otto Warburg over 60 years ago, was one of the first phenotypic
alterations recognized in cancerous cells that confers a survival advantage (59, 204, 215). Warburg took advantage of new techniques for simultaneously measuring gas consumption and lactate production in tumor slices either in the presence or absence of oxygen. Surprisingly, in the presence of oxygen, the rapidly growing tumor cells consumed glucose at a higher rate and secreted elevated amounts of lactate compared to normal cells (204). More recently, Positron Emission Tomography (PET) imaging technique of fast-growing and metastatic human cancers using the glucose analogue tracer 18-fluorodeoxyglucose (FDG) has confirmed Warburg's observations (57, 119, 129).

The first compelling evidence that mitochondrial dysfunction was associated with tumorigenesis was presented less than a decade ago when mutations in succinate dehydrogenase (SDH) or fumarate hydratase (FH), both enzymes of the TCA cycle, were found to be initiating events of familial paraganglioma or leiomyoma and of papillary renal cell cancer, respectively (10, 188). To demonstrate that mtDNA mutations can also contribute to the oncogenicity of tumors, a known pathogenic mtDNA mutation was introduced into the prostate cancer cell line PC3, which resulted in increased tumorigenicity (143). Furthermore, neoplastic transformation has been found to produce marked changes in the expression of both nuclear and mitochondrial OXPHOS genes (133, 190). These changes have been also proposed to occur
in tumors developed in a tissue-specific fashion (14, 27, 198). A study performed in *Caenorhabditis elegans* showed that inactivation of the *Cytb* (a mtDNA-encoded gene) subunit of complex II markedly increases mitochondrial ROS production (72). Thus, ROS production appears to be the factor that links mitochondrial defects to neoplastic transformation and metastasis (74, 210). However, the precise mechanism by which mitochondrial RNA and/or ROS levels increase in cancerous cells still remains unclear (35).

On the other hand, the direct role that mitochondria play in apoptosis has been extensively studied during the past two decades. For instance, loss of mitochondrial membrane potential is thought to contribute to cell death by disruption of normal mitochondrial function (20, 76, 96). Further, mitochondrial outer membrane permeabilization (MOMP) leads to the release of proapoptotic factors such as cytochrome c and AIF. Therefore, several MOMP inhibitors (e.g. Bcl-2, Bcl-XL, VDAC isomers, hexokinase II) have been described and are currently targeted in the chemotherapeutic treatment of cancer (3, 53, 203).
Objectives

In comparison to the immunocompetent population, AIDS and post-transplant patients have a significant increase of developing virus-induced cancers. Lymphoproliferative disorders (LD) in particular, have been found to be diagnosed more frequently than other types of neoplasias. Nevertheless, it is still unknown why some LD subtypes are more prevalent than others in AIDS versus post-transplant patients. The discovery of new viruses associated with cancer during the last two decades and the immunosuppressive state of these individuals suggest that unidentified infectious agents might be responsible for inducing neoplastic transformation within this context.

Since γ-herpesviruses (e.g. KSHV and EBV) have been directly linked to specific cases of LD in humans (16, 136, 166), one of our main goals was to determine whether EBV- and KSHV-negative AIDS-associated lymphomas could be caused by a large DNA virus. Since γ-herpesviruses are maintained as circularized extrachromosomal nuclear genome copies during latency (37), it was investigated whether these LD samples could contain novel episomal
DNA. Despite the fact that no novel DNA was detected in the samples tested, dimers and concatemers of the mitochondrial genome were identified. Furthermore, independent of HIV infection, established lymphoma cell lines also harbored this type of mtDNA. In this dissertation, a detailed analysis of the mtDNA concatemers identified in a case of AIDS-associated lymphoma (EL) is presented. This work also shows evidence that formation of mtDNA concatemers and dimers are associated with neoplastic transformation by immortalizing normal human T-cells with Herpesvirus \textit{saimiri}. Further studies on this area might reveal a new avenue for the therapeutic treatment of lymphoma.

The following aims were designed for this dissertation project:

1. To characterize the cccDNA identified in a case of EBV- and KSHV-negative AIDS-associated lymphoma (EL).

2. To determine if mitochondrial genome concatemers can influence mitochondrial and cellular function.

3. To determine if mitochondrial genome concatemers are associated with immortalization of normal human T-cells with Herpesvirus \textit{saimiri}.
Materials and methods

Primary AIDS-associated lymphomas and human T-cell samples

Twelve non-Hodgkin’s lymphoma samples from HIV-positive patients were obtained through the Division of Cancer Treatment and Diagnosis at the National Cancer Institute. Samples were cultured in RPMI 1640 medium supplemented with 4 mM L-glutamine, 10% fetal bovine serum and 50μg/ml gentamycin. Peripheral blood mononuclear cells were isolated from blood bank donor samples and T-cell cultures were obtained by PHA stimulation (10 μg/ml) of mononuclear cells for 3 days, then cultured in medium containing 50U/ml of IL-2.

Method for detection of cccDNA

Live lymphoid or AIDS-associated lymphoma cells were selected by Lymphoprep™ (ProGen), according to manufacturer's protocol. 1x10⁶ cells were loaded in each well of a vertical 0.8% agarose gel in 10% Ficoll buffer (54). Cell lysis buffer containing 5% Ficoll, 1% SDS, 1mg/ml pronase and
0.05% xylene cyanol green was layered on top of the cell samples. Electrophoresis was carried at 0.8 V/cm for 3 h, then increased to 7.5 V/cm for 15 h at 4°C. The gel was stained with 20 µl of SYBRGreen™ Nucleic Acid Stain (Lonza Rockland, Inc., Rockland, ME) diluted in 200 ml of 1X TBE buffer for 1 hr. Then, the gel was de-stained in 10 mM Tris, 1 mM EDTA for 30 minutes and the DNA was visualized using a Molecular Dynamics STORM Phosphor Imager (Model 860-PC, Amersham Biosciences, Piscataway, NJ).

**Southern blot analysis**

Agarose gels containing electrophoresed DNA were consecutively submerged and gently rocked twice for 15 min each in the following solutions: 1) 0.25 M HCl solution for DNA depurination, 2) 1 M NaCl, 0.5 M NaOH solution for DNA denaturation and residual RNA removal, and 3) a neutralizing solution containing 0.5 M Tris-HCl (pH 7.0), 1.5 M NaCl.

Treated gels were vacuum-blotted onto nitrocellulose membranes (125). After blotting, the DNA was immobilized on the membrane by baking for 1 h in a vacuum oven at 80°C. At this point, the membrane could be either used for hybridization or stored at -20°C. 10 ml of a prehybridization buffer is added to the membrane at RT for 10 min. The prehybridization buffer contains 50% formamide, 5X SSC (750 mM NaCl, 75 mM sodium citrate), 10X Denhardt’s
solution (0.2% bovine serum albumin, 0.2% Ficoll 400 and 0.2% polyvinylpyrrolidone), 0.05 M Na$_3$PO$_4$, 0.5 mg/ml salmon sperm (or total E.coli DNA), and 0.1% SDS. 10 ml of hybridization buffer are heated at 100°C for 5 min then cooled down to RT. The hybridization buffer consists of 50% formamide, 5X SSC, 1X Denhardt’s solution, 0.015 M Na$_3$PO$_4$, 0.1 mg/ml salmon sperm, 5% dextran sulfate and 0.1% SDS. Then, prehybridization buffer is removed; the radiolabeled probe is added to the hybridization buffer, mixed and added to the membrane. Hybridization is performed for 16-18 h at RT. The membrane is then washed at RT for 3-4 times with a solution containing 0.1% SDS and 0.1X SSC. Finally, the membrane is exposed for autoradiography for 16-24 h to a Phosphor Screen and analyzed using a Molecular Dynamics STORM Phosphor Imager (Model 860-PC, Amersham Biosciences, Piscataway, NJ).

**Synthesis of a [γ$^{32}$P]-ATP-radiolabeled probe by 5’-end labeling of DNA oligonucleotides**

Human mtDNA-derived oligonucleotides (Table 2) were mixed, at a final concentration of 10 µg/ml, in 1X TE buffer (primer mix). Then, the radiolabeled probe was made by adding in an eppendorf tube 1µl of primer mix to 1µl of 10X T4 polynucleotide kinase buffer, 1 µl (10U) of T4 kinase and 7 µl of [γ$^{32}$P]-dATP isotope. The mixture was incubated for 30 min at 37°C. Then, 40 µl of a
solution (stop buffer) consisting of 20 mM NaCl, 50 mM EDTA, 20 mM Tris-HCl (pH 7.5), 1% SDS and 0.25% dextran blue, were added. The radiolabeled probe was kept at RT for hybridization.

Isolation of cccDNA by alkaline cell lysis and CsCl-EtBr gradient

5x10^8 cells were centrifuged at 500 x g for 15 min and resuspended in 10 ml 1X PBS. Five ml of an alkaline buffer solution containing 50 mM NaCl, 2 mM EDTA, 1% SDS and 30 mM NaOH were added to the cells and the mixture was vortexed for 2 min and incubated at 30°C for 30 min. Then, the mixture was neutralized by addition of 80.4 μl of 1 M Tris (pH 7.0) and 200 μl of 5 M NaCl. Proteinase K (60 μg/ml) was added and mixture was incubated at 37°C for 30 min. DNA was extracted with an equal volume of 24:24:1 phenol:chloroform:isoamyl alcohol solution followed by extraction with chloroform. DNA was then precipitated by centrifugation at 20,000 rpm at 4°C with 2.5 volumes of 95% ethanol. DNA was air-dried and pellet was resuspended in a 1X TE solution. Then, cesium chloride (CsCl) (1.55 g/ml final density) and ethidium bromide (EtBr) (1μg/ml final concentration) were added. The mixture was centrifuged at 45,000 rpm in a Beckman L7-75, VTi65 rotor for 72 h at 20°C. After 3 days, 200 μl fractions from higher density (bottom of the tube) to lower density (top of the tube) were collected in a 96-well plate and aliquots from each fraction were analyzed by electrophoresis in a 0.8% agarose
gel. Fractions corresponding to cccDNA were further purified by repeated extractions with 1 volume 5M NaCl isopropanol. The mixture containing the cccDNA (stripped of EtBr) was mixed with 2.5 volumes of 95% ethanol, centrifuged at 20,000 x g for 30 min at 4°C, and the pellet was resuspended in 1X TE buffer.

**Cloning of fragments from EL cccDNA**

Purified EL cccDNA was electrophoresed in a 0.8% low-melting temperature agarose gel for 18 h at 22V, 4°C. The gel was stained with SYBRGreen™ and DNA visualized in a UV-box. The band corresponding to high MW cccDNA was cut out from the gel and incubated at 65°C for 10 min. The cccDNA was purified by phenol/chloroform extraction and ethanol precipitation. Cloning of EL cccDNA was performed by restriction digestion using the four-base-cutter endonuclease *Mbo* I followed by T4-ligation to *Bam* HI-digested and dephosphorylated pBluescript® II KS vector. Ligation mixture was phenol/chloroform extracted, ethanol precipitated and resuspended in 10 µl of nuclease-free water.
Electroporation of competent *E. coli* and selection of transformants

1 µl of ligated DNA was mixed with 10 µl of competent *E. coli* (ElectroMAX DH10B strain) and incubated on ice for 10 min. The suspension was put in a 1-mm electrode-gap cuvette and electroporated at 1,500 V using an Eppendorf Electroporator 2510. Cells were incubated for 1h at 37°C in 500 µl of SOC medium (20 g/L bacto-tryptone, 5 g/L yeast extract, 0.5 g NaCl, 2.5 mM KCl, pH 7.0). Different dilutions of electroporated cells to SOC medium (1:1, 1:10 and 1:100) were plated in agar plates containing ampicillin (100 µg/ml final concentration), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to a final concentration of 80 μg/ml (prepared in dimethylformamide, DMF), and isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 20 mM (prepared in sterile ddH2O). Finally, the plates were incubated overnight at 37°C and transformants containing EL cccDNA fragments were selected by blue-white color screening.

PCR analysis

Purified cccDNA was electrophoresed in a low-melting temperature 0.8% agarose gel and ran overnight at 4°C and 22 V. After 16 h, gel staining and DNA visualization in a UV-box were performed. The gel fractions containing cccDNA for both monomers and concatemers were cut out and melted at 65°C for 10 min, then cccDNA was phenol/chloroform extracted and ethanol
precipitated. Oligonucleotides were used either for amplification or as probes for Southern blotting (Table 2) (151): PCR products were electrophoresed in a 0.8% agarose gel to confirm size (in comparison to predicted size from official human mtDNA sequence). Then, PCR products were sequenced and analyzed using CLC Workbench 3®.

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Table 2. Oligonucleotides used for PCR amplification and sequencing of EL cccDNA and mtDNA of normal T-cells.

3’-end positions are relative to the revised Cambridge reference sequence of the human mtDNA available at www.mitomap.org.
DNA sequencing

We submitted all EL cccDNA-derived clones and purified PCR products for sequencing to the Molecular Biology core facility at the H. Lee Moffitt Cancer Center and Research Institute.

Electron microscopy

Mitochondrial DNA samples were prepared for electron microscope imaging using a modified Kleinschmidt protocol (99, 213). DNA was mixed with ammonium acetate and cytochrome C, placed on a piece of parafilm to allow a protein monolayer to form. A carbon coated electron microscopy grid is put in contact with the surface of the sample. Grids were washed with a solution of 95% ethanol/50 uM uranyl acetate. Grids were then either visualized as is or rotary metal shadowed with tungsten in an Edwards Auto 306 vacuum evaporator. The length of the DNA molecules was confirmed by comparison to plasmids of known length. The grids were imaged in a Hitachi H7500 TEM equipped with an Advanced Microscopy Techniques XR60 CCD camera. The NIH Image J was used to trace the backbone of the DNA molecules and the DNA length was calculated (EM was performed by Troy Lund and Stephen Jett).
Flow cytometry analysis for characterization of EL cells

$1 \times 10^6$ EL cells and normal T-cells for controls, were resuspended in 5ml of staining solution containing 1X PBS, 0.1% (w/v) NaN₃ and 1.0% (w/v) bovine serum albumin (BSA; fraction V) on ice. Then, cells were fluorescently labeled using staining media containing 10 µl of each of the FITC-conjugated antibodies used and isotype (IgG1 and IgG2) for controls, diluted to the appropriate concentrations. Cell suspensions were analyzed in a BD FACSCalibur™ Flow cytometer. Monoclonal mouse or goat anti-human antibodies for the following markers were used: CD2, CD15, CD45, CD8, CD10, CD19, CD20, CD22, CD33, HLA-DR, CD13, CD14.

Flow cytometry analysis of mitochondrial factors and properties

1. Mitochondrial membrane potential ($\Delta\psi_m$)

$\Delta\psi_m$ was estimated by staining for 15 min at 37°C with cationic lipophilic dye 20 nm 3,3'-dihexyloxacarbocyanine iodide (DiOC6; Molecular Probes, Eugene, OR; excitation: 488 nm, emission: 525 nm). Fluorescence of DiOC6 is oxidation-independent and correlates with $\Delta\psi_m$ (182). $\Delta\psi_m$ was also quantitated by using a potential-dependent J-aggregate-forming lipophilic cation, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazocarbocyanine iodide (JC-1) (173). JC-1 selectively incorporates into mitochondria, where it forms
monomers (fluorescence in green, 527 nm) or aggregates, at high
transmembrane potentials (fluorescence in red, 590 nm) (40, 118, 173). Cells
were incubated with 0.5 μM JC-1 for 15 min at 37°C before flow cytometry.
Δψm changes were also confirmed by staining with 1 μM CMXRos (excitation:
579 nm, emission: 599 nm) and 1 μM tetramethyl rhodamine methylester
perchlorate (TMRM) (excitation: 543 nm, emission: 567 nm; Molecular Probes).
Cotreatment with a protonophore, 5 μM carbonyl cyanide m-
chlorophenylhydrazone (mClCCP; Sigma) for 15 min at 37°C resulted in
decreased DiOC6, CMXRos, TMRM, and JC-1 fluorescence and served as a
positive control for disruption of Δψm (9).

2. Mitochondrial mass

Mitochondrial mass was monitored by staining with potential-insensitive
mitochondrial dyes 50 nM nonyl acridine orange (NAO, excitation: 490 nm,
emission: 540 nm) or 100 nM MitoTracker green-FM (excitation: 490 nm,
emission: 516 nm; Molecular Probes). Samples were analyzed on a Becton
Dickinson Vantage S/E flow cytometer equipped with an Enterprise II argon
laser producing two laser lines (emission of 351-364 nm at 60 mW and
emission of 488 nm at 350 mW) and a Coherent (Santa Clara, CA) 70C
Spectrum krypton-argon laser operated at 200 mW (emission at 647 nm).
Dead cells and debris were excluded from the analysis by electronic gating of
forward scatter (FSC) and side scatter (SSC) measurements. Each measurement was carried out on 10,000 cells.

3. Production of ROS

Intracellular amounts of ROS were assessed fluorometrically using oxidation-sensitive fluorescent probes 5,6-carboxy-2',7'-dichlorofluorescein-diacetate (DCFH-DA), dihydrorhodamine 123 (DHR), and dihydroethidium (hydroethidine, DHE; Molecular Probes, Eugene, OR) as described (8, 9). Cells were incubated with 0.1 μM DHR for 2 min, 1 μM DCFH-DA for 15 min, or 1 μM DHE for 15 min. Fluorescence emission from 5,6-carboxy-2',7'-dichlorofluorescein (DCF; green) or DHR (green) was detected at a wavelength of 530 ± 30 nm. Fluorescence emission from oxidized hydroethidine ethidium (red), was detected at a wavelength of 605 nm. While R123, the fluorescent product of DHR oxidation, binds selectively to the inner mitochondrial membrane, the oxidized products of DCFH-DA and DHE, ethidium and dichlorofluorescein (DCF), respectively, remain in the cytosol of living cells. DCF and DHE preferentially detect H₂O₂ and superoxide (O₂⁻), respectively (19, 90, 153).
4. Glutathione levels

Intracellular glutathione levels were assessed with 100 μM monochlorobimane (excitation: 380 nm/emission: 461 nm) following similar protocols for cell staining and sample measurements as in ROS assays.

5. Fas-induced apoptosis

Apoptosis was induced with Fas Ab CH-11 (MBL, Watertown, MA). Briefly, twenty-four hours before assays, cells were fed fresh medium and seeded at a density of 2 x 10^5 cells/ml, and cell death was induced with 50 or 100 ng/ml anti-Fas mAb CH-11. Apoptosis was monitored by observing cell shrinkage and nuclear fragmentation and was quantified by trypan blue exclusion (191). DNA fragmentation during apoptosis was monitored by agarose gel electrophoresis (8). Apoptosis was also measured by flow cytometry after concurrent staining with fluorescein-conjugated annexin V (annexin V-FITC; R&D Systems, Minneapolis, MN) and propidium iodide (122, 193). Staining with phycoerythrin-conjugated annexin V (annexin V-PE; R&D Systems) was used to monitor phosphatidyl serine (PS) externalization in parallel with measurement of ROS levels and Δψm, using DHR, DCF, or DiOC6 fluorescence, respectively. Staining with annexin V alone or in combination with DHR or DiOC6 was conducted in 10mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂.
Immortalization of normal human T-cells using Herpesvirus saimiri

A monolayer of owl monkey kidney (OMK) cells grown in a 100 mm-dish were infected with $1 \times 10^5$ pfu/ml of Herpesvirus saimiri (HVS) strain 484-77 for virus propagation (127). After 1h post-infection, supernatant was removed. Human peripheral blood leukocytes (PBL) were isolated from blood samples by density gradient sedimentation using Lymphoprep™ (ProGen) according to manufacturer's instructions. Then, PBL were cultured in RPMI 1640 cell media containing 10% fetal calf serum (FCS), 4 mM L-glutamine, 50 μg/ml gentamycin and 0.5 ug/ml of phytohemagglutinin (PHA) for 3 days. OMK cells showing cytopathic effect were overlaid with human T-cells. Cells were maintained in complete media with or without 20U/ml of IL-2 for around two more months (181). Although control uninfected cultures usually died around 3-5 weeks post-PHA stimulation, some infected PBL cultures were successfully expanded (e.g. T-484 and 3HB6). After removal of IL-2, these cultures continuously grew and were selected for analysis.
Results

Method for detection of large cccDNA of AIDS-associated lymphomas

To visualize cccDNA from tumor samples suspensions the vertical gel electrophoresis method of Gardella et al. was modified (54). One million cells from each of the twelve AIDS-associated lymphoma samples were run in a Gardella gel and the gel was stained with SYBRGreen™ (Figure 6). In some of the samples, DNA bands migrating between the loading well and linear broken DNA were observed; this range of these gels contains large (15-250 kb) cccDNA. Four AIDS-associated lymphoma bands were positive for EBV by Southern hybridization (not shown) and no longer studied. The remaining eight samples were also negative for KSHV.

Identification of large cccDNA in an AIDS-associated lymphoma (EL) that does not hybridize with EBV- or KSHV-derived probes

To further study the eight EBV- and KSHV-negative lymphomas, formation of permanent cell lines was carried out. After a period of around 3 months in culture, one lymphoma sample (denominated EL) successfully
Figure 6. The Gardella gel technique and visualization of large cccDNA in an AIDS-associated lymphoma sample (EL).

One million live cells in 10% Ficoll buffer are loaded into a well of a vertical agarose gel and digested by overlaying with a lysis buffer containing SDS and pronase followed by electrophoresis at 22V for 3 h. Then, electrophoresis is set at 70V for 18 h. Finally, the DNA is visualized by staining with SYBRGreen™. The three different molecular configurations of DNA are indicated. Right panel shows SYBRGreen™ staining of Gardella gel containing unknown episomal DNA from EL cells (arrow) migrating within the range of large cccDNA.
yielded a cell line while all other EBV/KSHV negative lymphoma samples eventually died. Gardella gel analysis of EL cells and the derived cell line consistently demonstrated the presence of a large cccDNA unrelated to EBV or KSHV (Figures 6 and 7).

**Phenotypic characterization of EL cells by flow cytometry**

EL cells were positive for CD3, CD4 and CD45, meaning that they are a CD4+ T-cell line. Other markers tested were: CD8 (specific for CTLs), CD16 (specific for NK cells, neutrophils, monocytes, and macrophages), CD19, CD20, CD22 (for B-cells), CD33 (for myeloid cells) and HLA-DR (for APCs). IgG1 and IgG2 were used as isotype controls (Figure 8).

**The EL cccDNA consists of mitochondrial DNA**

To determine the sequence of the EL cccDNA, the cells were expanded in RPMI 1640 10% FCS without IL-2. CccDNA was isolated from EL cells by alkaline lysis followed by centrifugation on a CsCl-EtBr density gradient. Fractions of the gradient were collected and separated by low melting agarose gel electrophoresis. Figure 9 shows that the higher CsCl density fractions corresponding to cccDNA contained two discrete bands and the lower density fraction contained smeared linear nuclear DNA as expected.
Figure 7. Southern blotting analysis of EL cccDNA using probes specific for both EBV and KSHV.

Total DNA from Gardella gels was transferred onto nitrocellulose membranes for Southern blotting. Radiolabeled probes specific for (A) EBV or (B) KSHV DNA do not hybridize with the large cccDNA identified in EL cells.
Figure 8. EL cells are CD4+ T-lymphocytes.

Phenotypic characterization of EL cells using flow cytometry analysis shows that EL cells are CD4+ T-lymphocytes. Other markers tested: IgG2, CD8, CD16, CD19, CD20, CD22, CD33, HLA-DR.
Figure 9. Gel electrophoresis analysis of CsCl-EtBr gradient fractions of total EL DNA.

Episomal cccDNA was purified from EL cells by alkaline lysis and CsCl–EtBr gradient centrifugation. Density of gradient fractions decreases from left to right. Fractions were analyzed by agarose gel electrophoresis followed by SYBRGreen™ staining. Figure is representative of 7 independent experiments. *High molecular weight genomic DNA; **genomic broken linear DNA front.
DNA was extracted from the molten gel slice corresponding to the two cccDNA bands and digested with restriction endonuclease *MboI*. The restriction fragments were shotgun-cloned into the pBluescript™ vector (Stratagene) and the clones were sequenced and analyzed using BLAST (Figure 10). Results indicated that the clones contained 95% (>300 clones) human mtDNA and ~5% (~20 clones) human chromosomal DNA randomly derived from most human chromosomes, presumably caused by contamination from nuclear DNA during collection of fractions (Table 3). Then, normal T-cells were obtained from healthy blood donors and cccDNA was isolated and compared with EL DNA. Gel electrophoresis shows that only one band was detected in normal T-cells while EL cccDNA contained two bands (Figure 11A). Southern hybridization of this gel using a human-mtDNA probe shows co-migrating bands (slightly above the 23 kb marker) in both normal T-cell and EL DNA. A slower-migrating cccDNA from EL cells also hybridized with the mitochondrial probe (Figure 11B). These results support our previous data that both forms of EL cccDNA were comprised of mtDNA. Moreover, the data show that EL cells contain some monomeric mtDNA and a larger and more abundant DNA hybridizing with the mtDNA probe.
Figure 10. Schematic representation of the method performed for isolation and analysis of EL cccDNA.

5 x 10^8 live cells in 10% Ficoll buffer are digested in an alkaline buffer. A solution containing proteinase K is added to the lysate. Total DNA is purified using phenol/chloroform extraction and ethanol precipitation. Purified DNA is then mixed with a solution containing CsCl and EtBr, and ultracentrifuged at 40,000 x g for 72 h. At the end of the centrifugation, the bottom of the tube is perforated with a sterile needle and fractions (from high to low density) are collected in a 96-well plate. An aliquot from each fraction is then loaded and run in an agarose gel. The gel is stained using SYBRGreen™ and the fractions containing cccDNA are identified and further purified for cloning.
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Table 3. DNA sequencing results of EL cccDNA cloning.
Figure 11. Gel electrophoresis and Southern blotting of purified cccDNA from EL and normal T-cells using mtDNA-derived probes.

A) SYBRGreen™ staining of purified total cccDNA fractions from EL and normal T-cells.

B) Southern blotting of DNA (from A) using a probe of radiolabeled human mtDNA-specific oligonucleotides.
The EL cccDNA is formed by tandem duplicated mitochondrial genomes.

Electron microscopy analysis of 50 molecules from purified EL circular episomal DNA showed superhelical DNA with an average backbone contour length of 10.3 μm (Figure 12). Compared to the human mitochondrial genome (contour length of 5 μm) the cccDNA of EL cells consisted mainly of molecules equivalent to duplicated mtDNA.

To confirm that the EL episomal DNA is formed by duplicated tandem repeats, inverted repeats or rearranged mitochondrial genomes, full and partial digestion using BamHI (a mtDNA-single-cutter restriction enzyme) was performed. Figure 13 shows that complete digestion of EL cccDNA with BamHI yields one 16.5 kb band. Partial digestion yielded two linear bands of approximately 16.5 and 33 kb corresponding to putative monomers and dimers of mtDNA. These results confirm our electron microscopy and sequencing data that the EL episomal DNA is conformed mainly by duplicated mitochondrial genomes organized in head-to-tail tandem orientation. Several additional restriction enzyme digestion data also support this conclusion (not shown).
Figure 12. Electron microscopy of EL cccDNA.

The contour length of the concatemeric mtDNA (EL cccDNA) was measured using the Image Processing and Analysis in Java (ImageJ) software from the NIH. DNA contour length was calculated to be around 10.3 µm. Similar results were obtained measuring the contour length of 50 other molecules. Note: EM was performed by Troy Lund and Stephen Jett.
Figure 13. Complete and partial BamHI-restriction digestion of EL cccDNA.

Banding pattern of fully digested EL cccDNA with BamHI (10U) (mtDNA single cutter) at 30, 60 and 90 min resembles that of linear human mtDNA monomer (16.5 kbp). Right panel shows partial digestion of EL cccDNA with BamHI (0.1U) stopped at increasing time points (5 s, 10 s, 20 s, 40 s, 1 min, 2 min, 5 min, 10 min, 20 min, 40 min, 1 h). After 5 min, two bands of linear DNA are observed representing putative human mtDNA monomers and dimers (16.5 and 33 kbp respectively).
Cytosine insertions are present in the control region of the EL mtDNA

To determine whether the EL mtDNA has suffered minor mutations, PCR amplification of purified EL cccDNA followed by DNA sequencing were performed using specific mtDNA derived oligonucleotides covering the entire mitochondrial genome (Table 2). PCR products of EL and normal T-cell DNA were indistinguishable by agarose electrophoresis (not shown) indicating absence of large insertions or deletions.

The D-loop, which is the main regulatory region of mtDNA replication and transcription, has a highly-conserved C-stretch sequence (nt 311 to 315) known as the conserved sequence box II (CSBII) that has been reported as a mutational hotspot in primary tumors (158). Figure 14 shows that sequencing of PCR-amplified EL mtDNA revealed two additional cytosine residues in the monomer and in the dimer within the CSBII near the primer of mtDNA replication. Normal T-cells did not contain additional cytosines in the CSBII region as shown in the human mtDNA reference sequence.
Figure 14. Multiple sequence alignment analysis of conserved sequence box II (CSBII) regions from EL and normal mtDNA.

Multiple DNA sequence alignments of EL cccDNA (mtDNA concatemers and monomers) and normal mtDNA were performed using ClustalX. Shown, the regions from the D-loop conserved sequence box II (CSBII, ranging nts 297-321) from 

\[
\begin{align*}
    EL (C) & : \text{EL mtDNA concatemer} \\
    EL (M) & : \text{EL mtDNA monomer} \\
    \text{Normal T-cells} (M) & : \text{normal T-cell mtDNA} \\
    \text{Ref. Seq. (NCBI)} & : \text{human mtDNA reference sequence from NCBI}
\end{align*}
\]

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Multiple sequence alignment analysis of conserved sequence box II (CSBII) regions from EL and normal mtDNA.
Functional and structural mitochondrial properties of EL cells

Figure 15 summarizes various properties of EL cell mitochondria. In comparison with Jurkat cells (selected due to reproducibility throughout experiments and phenotypic similarity to normal T-cells (8)). EL lymphoma cells showed increased ROS and peroxide-induced apoptosis as determined by staining with dihydroethidium (DHE) and AnnexinV-Alexa 647, respectively. These results suggest an unstable OXPHOS system in EL cells, since ROS could leak through uncoupled mitochondrial complexes (I-V), thus augmenting intrinsic pro-apoptotic signals. In contrast, monitoring of TMRM, NAO and AnnexinV-Alexa-647 fluorescence indicated that mitochondrial membrane potential, mitochondrial mass and Fas-mediated apoptosis were respectively reduced in EL cells. Also, glutathione levels were significantly reduced in EL cells, which could explain the overproduction of ROS in these cells since glutathione works as a biological ROS scavenger (64).

Mitochondrial genome concatemers are present in primary AIDS-associated lymphomas and lymphoma cell lines

Primary AIDS-associated lymphomas and lymphoma cell lines were screened for episomal cccDNA using the Gardella technique (Figure 16). Southern hybridization detected two bands in control T-cells; a broader and slower migrating circular form and a faster migrating linear form. However, all
Figure 15. Flow cytometric analysis of mitochondrial physiological and structural properties.

Reactive oxygen intermediates (ROI), Fas-induced apoptosis, mitochondrial mass, mitochondrial potential and glutathione (GSH) levels in EL lymphoma cells relative to Jurkat T-cells used as controls (8). Data represent mean ± SE of 4 independent experiments. * P-value<0.05.
Figure 16. Mitochondrial genome concatamers are present in primary AIDS-associated lymphomas and lymphoma cell lines.

Primary AIDS-associated lymphoma samples, various lymphoma cell lines and normal peripheral T-cell controls were analyzed by the method of Gardella et al. (54). Southern blotting analysis was performed by using human mtDNA-specific radiolabeled primer probes. Samples: AIDS-associated lymphomas (2F7, 98-001, 98-002, EL); human body cavity lymphoma tumor cell line positive for KSHV (BCBL-1); B-cell Burkitt’s lymphoma (Raji); H. saimiri-induced rabbit lymphoma (484Th) (126); non-Hodgkin’s lymphoma positive for EBV and HIV (EBV-HIV-NHL).
lymphoma cell lines contained an additional band co-migrating with EL cccDNA. Therefore, all lymphoma cells encode both monomer and concatemer forms of mitochondrial genomes. Small amounts of broken linear mtDNA were also present in all samples probably due to dying of some cells. The linear mitochondrial bands co-migrated with controls indicating lack of tandem duplication of mtDNA. In contrast, EL cells contained a unique larger and very strong DNA band, and a faint smaller linear mitochondrial genome. The smaller one was co-migrating with bands from all other cells confirming the presence of small amount of monomers in EL cells. Taken together, all lymphoma cell lines encode both mitochondrial genome monomers and concatemers consisting of two monomers probably due to interlinking of two unit-length mitochondrial circles.

Total cccDNA was also purified by CsCl-EtBr density gradients from established human lymphoma cell lines such as K562 (a chronic myeloid leukemia cell line), Raji (an EBV-positive Burkitt’s B-cell lymphoma) and Molt3 (an acute T-cell lymphoma cell line). Results show that these cell lines also contain mtDNA concatemers, although different monomer/concatemer ratios were observed for each sample. An additional band running lower than monomeric mtDNA was obtained in Raji, possibly caused by the presence of EBV. A bar diagram using relative units from densitometric analysis of the gel shows monomer and concatemer amounts in comparison to EL (Figure 17).
Figure 17. Gel electrophoresis and densitometric analysis of cccDNA from human lymphoma cell lines.

Total cccDNA was isolated from EL and established cell lines by alkaline cell lysis and CsCl-EtBr density gradients. 5 µl of purified cccDNA from each sample was electrophoresed in an 0.8% agarose gel for 16 h and stained using SYBRGreen™, then visualized in a UV-light box. CccDNA bands (monomers or concatemers) were quantified by densitometric analysis using Image J and plotted on a bar diagram according to relative densitometric units.
Mitochondrial genome concatemers are associated with oncogenic transformation of lymphoid cells

Gardella gel and Southern blotting analyses show that *H. saimiri*-immortalized human T-cells (T-484 and 3HB6) contain DNA bands hybridizing with the mtDNA-derived radiolabeled probe. Some bands migrated slower than the wild-type mitochondrial genome, representing mtDNA concatemers. A much fainter band was also detected for normal T-cells (Figure 18).

Densitometric unit mean values between monomers *versus* concatemers present per cell sample were statistically significant (P-values of 0.009 for T-cells, 0.03 for T-484 and 0.06 for 3HB6). Also, mean values between concatemers present in T-cells *versus* immortalized cell lines were significant (P-values <0.05) (Figure 19).

Statistical analysis

Student’s t-tests were performed to evaluate relative units from flow cytometry assays of EL *versus* control cells to determine significance level of variation in mitochondrial function and factors, and to compare densitometric unit mean values of monomers and concatemers, and of concatemer relative mean value units in normal *versus* *H. saimiri*-immortalized T-cells. A p-value<0.05 was considered as significant.
Figure 18. Southern blotting assay of Gardella gel of Herpesvirus *saimiri*-immortalized human T-cells reveals the presence of mitochondrial genome concatemers. Mitochondrial genome concatemers were identified in Herpesvirus *saimiri*-immortalized T-cells (T-484 and 3HB6) using the Gardella technique followed by Southern blotting using mtDNA-derived oligonucleotides. Figure is representative of 4 independent experiments.
Figure 19. Densitometric analysis of mitochondrial DNA from Herpesvirus-saimiri-immortalized and normal T-cells.

Mitochondrial DNA bands (from Gardella gels) of Herpesvirus saimiri-immortalized (T-484 and 3HB6) and normal T-cells were analyzed by densitometry. Shown, mean ± S.E., and P-values of monomer and concatemer mean values for each sample, and of concatemer relative mean value units for immortalized versus normal T-cells.
Discussion

Identification of mitochondrial genome concatemers in lymphomas

In this study, a method was developed for the visualization of cccDNA in mammalian cells based on a cell lysis method previously described (54). This technique requires only $1 \times 10^6$ cells and is ideal for screening large number of cell samples. It is rapid, reliable and can be concurrently harnessed for the detection of existing or novel episomes of viruses and for the identification of size alterations such as insertions, deletions and concatemerization of mtDNA.

Mitochondrial genome concatemers were identified in primary tumor suspension samples obtained from AIDS-associated lymphoma patients and in seven established mammalian lymphoma cell lines. These concatemers were found to be conformed of two kinds. All lymphoma cells, except EL cells, contained mitochondrial genome concatemers as interlinked monomers. In contrast, most EL mitochondrial genomes are comprised of two unit length molecules joined in head-to-tail configuration as demonstrated by several independent methods such as Gardella gels, mtDNA complete and partial digestions, and electron microscopy (Figure 20).
Figure 20. Schematic model of mtDNA configurations identified in AIDS-associated lymphomas and lymphoma cell lines.

Normal T-cells contain mostly wild-type mtDNA (monomers of 16 kb), EL cells contain mostly duplicated mitochondrial genomes (dimers of ~32 kb), and concatemers (two or more interlocked mitochondrial genomes, ≥32 kb) were observed in AIDS-associated lymphomas and lymphoma cell lines analyzed.
Proposed molecular mechanisms of mtDNA concatemer formation

Although the specific molecular mechanism for mtDNA concatemer formation remains elusive, a considerable amount of evidence has been reported on processes associated with mtDNA maintenance and stability which might be responsible for the formation of mtDNA concatemers in transformed lymphoid cells. Two mechanisms were proposed: homologous recombination of mtDNA and mtDNA replication.

1) Homologous recombination of mtDNA

Homologous recombination (HR) occurs in all life forms, and is an important mechanism for the repair of damaged DNA and maintenance of chromosomal integrity (157, 178). In humans, several cancer-prone genetic diseases (e.g. Fanconi anemia and Bloom’s syndrome) are associated with HR dysfunction or deficiency (84, 178, 179). Moreover, mutations in the HR-mediators BRCA1 and BRCA2 genes have been associated with familial breast and ovarian cancer (75).

Although HR of the nuclear genome of eukaryotes has been extensively studied, much less is known about HR in mitochondria. MtDNA HR has been documented for a few organisms (50, 116, 117). In the yeast Saccharomyces cerevisiae, an active process of mtDNA HR has been demonstrated (111, 113).
It involves the participation of the mitochondrial homologous recombination protein 1 (Mhr1) in the formation of HR intermediates of mtDNA, by pairing ssDNA with homologous dsDNA forming heteroduplex joints (113), and the resolution of recombination junctions by the cruciform cutting endonuclease (Cce1) (88). Interestingly, Mhr1-dependent formation of concatemeric mtDNA was observed when generating mitochondrial homoplasmic yeast cells (112). However, mammalian homologues for either one of these proteins still remain to be identified.

A homologue of the bacterial recombinase RecA, targeted to mitochondria, has been identified in Arabidopsis thaliana and other eukaryotes (85, 137). Also, a RecA homologue targeted to chloroplasts with HR and repair activity was described (22). In humans, RecA homologues such as RAD51 and RAD52 have been cloned and are currently under intense investigation; however, despite their important role in nuclear HR, none of these proteins has been found in mitochondria (110). Hence, it has been hypothesized that other proteins or mechanisms responsible for mtDNA HR (or a similar process) must be present in mammalian mitochondria (4). Several reports have shown that HR can also occur in human tissues. Kraytsberg et al. identified heterologous (paternal and maternal) mtDNA in muscle tissue of an individual with paternal inheritance of the mitochondrial genome (93). Kajander et al. detected mtDNA HR intermediates in human heart muscle (78). Campbell and colleagues
demonstrated that a human mitochondrial protein related to RecA can catalyze HR of the mtDNA in vitro (186). Furthermore, spontaneously immortalized murine and human fibroblasts, and primate and murine established fibroblasts cell lines performed HR at frequencies around 100-fold higher than did normal cells (187). Therefore, formation of mtDNA concatemers might occur through genetic recombination of monomers of the mitochondrial genome, and represents an appealing model to examine within the lymphoma context.

In two seminal papers describing genetic recombination in E. coli, Potter H. et al. showed that DNA molecules of the plasmid ColE1 can be recovered from wild-type cells as a set of monomer- and multimer-size rings (145, 146). Moreover, they demonstrated that multimers are a product of genetic recombination since bacterial host cells lacking a functional RecA gene could no longer produce such DNA species (146). Finally, they proposed that multimers are the result of the maturation of Holliday intermediates formed between two monomer plasmid molecules (146).

The elucidation by Mootha and colleagues of a protein compendium for mammalian mitochondria showed that around 50% of the proteins that form the mitochondrial compartment do not have a bona fide mitochondrial targeting sequence (MTS) (141). Therefore, other mechanism(s) for protein translocation analogous to the TIM-TOM system must be present in these
organelles (130). The identification of such mechanisms might explain how mostly nuclear proteins such as p53 or STAT3 can take transient residence in mitochondria (120, 205). Thus, recombinases that function in the nucleus would also be able to translocate under certain circumstances (e.g. neoplastic transformation, oxidative stress, etc.) by the same mechanisms and induce the formation of concatemeric mtDNA through genetic recombination.

2) MtDNA replication

Two models for mammalian mtDNA replication have been proposed: the strand-displacement and the strand-coupled models (Figures 4 and 5) (31). The strand-displacement model was mainly determined by the analysis of replication intermediates using EM (34). Due to the presence of two separated origins of replication (OH for the heavy DNA strand, and OL for the light DNA strand), during mtDNA replication, two types of mtDNA progeny have been observed: The first one is a duplex circle with a newly synthesized leading strand, and the other is a gapped circle with a partially formed lagging strand (31). In both cases, the addition of nucleotides and strand ligation at the end of the process of mtDNA replication result in the formation of closed circular mtDNA monomer molecules. Therefore, a defective resolution of mtDNA replication intermediates into mature monomer molecules could result in an increase in the amount of concatemers. However, the presence of head-to-tail
dimers and concatemers in transformed cells would be more in accordance to
replication intermediates observed in the strand-coupled model (31, 70).
Simultaneous bidirectional migration of newly formed leading and lagging
strands would result in circular dsDNA dimeric or concatemeric intermediates
that would be then processed to form circular monomers in normal cells.

It is widely accepted that cells undergoing oncogenic transformation
have marked differences in mtDNA expression and content (68, 190). A
prospect study on mtDNA copy number and risk of non-Hodgkin’s lymphoma
(NHL) showed that mtDNA amounts are increased in patients with chronic
lymphocytic leukemia (CLL), in Burkitt’s lymphoma and Epstein-Barr virus-
transformed lymphoblastoid cell lines (97). Although mtDNA configuration was
not determined in this study, it is possible that increasing amounts of
concatemers were also the reason for the overall mtDNA increase detected in
these NHL samples.

Defects in several mtDNA-replication factors have been associated to
human disorders (38). Mitochondrial genetic diseases can result from defects
in mtDNA in the form of deletions, point mutations, or depletion. These
mutations may be spontaneous, maternally inherited, or a result of inherited
nuclear defects in genes that maintain the mtDNA (38, 39). It is possible that
formation of mtDNA concatemers results also from defects in one or more of these factors in cells undergoing oncogenic transformation.

Differential expression of A and B subunits of DNApol-γ, mitochondrial ssDNA-binding factor (MtssB) and the mtDNA helicase (Twinkle) has been reported in leukocytes and in leukemic cell lines in comparison to other human tissues (165) (Figures 21 and 22). Furthermore, mutations and SNPs in the gene encoding the catalytic subunit of the mtDNA polymerase, DNApol-γ, have been associated with such diverse disorders as progressive external ophthalmoplegia (PEO), parkinsonism, Alpers syndrome, mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), and sensory ataxic neuropathy, dysarthria, and ophthalmoparesis (SANDO) (38, 71, 115). However, to date, the effect that mutations or SNPs in DNApol-γ (or another factor associated with mtDNA replication) may have during neoplastic transformation has not been addressed.
Figure 21. DNApol-γ (subunit A and B) mRNA levels in several human tissues and human cell lines (GNF Human Gene Atlas).

Transcript levels of subunits A and B of DNA polymerase gamma (DNApol-γ) were determined for several human tissues and cell lines (ref. GNF Human Gene Atlas). Highlighted with brackets, mRNA levels of normal leukocytes and leukemic cell lines. To note, DNApol-γ mRNA levels are higher in leukocytes (normal and cell lines) than in any other human tissue analyzed.
Figure 22. MtssB and Twinkle mRNA levels in several human tissues and human cell lines (from GNF Human Gene Atlas).

Transcript levels of mitochondrial single-stranded DNA binding protein (MtssB) and mtDNA helicase (Twinkle) were determined for several human tissues and cell lines (GNF Human Gene Atlas). Highlighted with brackets, mRNA levels of normal leukocytes and leukemic cell lines. To note, MtssB and Twinkle mRNA levels are higher in normal leukocytes than in any other human tissue analyzed (including leukemic cell lines).
Cytosine insertions in the mtDNA control region

Within the mitochondrial genome, a single major non-coding region known as the D-loop, contains the main regulatory sequences for transcription and replication initiation (183). DNApol-γ initiates replication at the origin bound with the replication primer, and proceeds along one DNA strand displacing the other strand (26, 34). In EL mitochondrial DNA, one to two cytosine insertions were present at a highly conserved sequence block (CSB) within the D-loop, known as CSBII. The presence of the mtDNA replication primer next to the CSBII suggests that CSBII might be important during initiation/termination of mtDNA replication, serving as a possible docking site for DNApol-γ (46). Nevertheless, the lack of an effective method to study mtDNA mutations or insertions in vivo has hindered further investigation on this phenomenon, which may be attributed to random genetic drift or rapid clonal expansion occurring in cells undergoing oncogenic transformation. Interestingly, insertion of C residues in the CSBII has been described as a frequent event in mtDNA alterations in human tumors (158). It could be possible that a higher G-C content might affect dissociation of the RNA primer from the DNA, affecting termination of DNA replication. Also, addition of cytosine residues might increase the base-pairing binding strength of both DNA strands influencing mtDNA termination and/or release of nascent molecules from parental mtDNA.
Nucleoside analogues used in the treatment of AIDS affect mtDNA stability

Highly active antiretroviral therapy (HAART), which includes nucleoside analogue reverse transcriptase inhibitors (NRTIs) in combination with other agents, has changed AIDS from a lethal illness to a chronic, manageable disease (106). Within the list of NRTIs, azidothymidine (AZT), a thymidine analogue that inhibits HIV reverse-transcription, has been one of the most widely used antiretroviral drugs in the treatment of AIDS. Due to substrate competitive inhibition, AZT can also function as a DNApol-γ inhibitor (121, 144, 212) (Figure 23). AZT treatment could result in the induction of impaired mtDNA replication that could promote the formation of mitochondrial genome concatemers and/or other mtDNA abnormalities, which may be associated to oncogenic transformation in individuals with AIDS. It is also recognized that mitochondrial functions are affected as a result of NRTI therapy (109). Cardiac dysfunction, hepatic failure, skeletal myopathy, lactic acidosis with defective mtDNA replication, mtDNA depletion and altered mitochondrial ultrastructure have been observed in selected tissues in vivo and in post-HAART patients (105, 107).
Figure 23. Inhibition of DNA polymerase gamma by NRTIs (AZT).

Diagram shows the structure of the mitochondrion. The nucleoside reverse transcriptase inhibitor, AZT, is phosphorylated by thymidine kinase 2 (TK2), the mitochondrial isoform, thus initiating a series of steps that lead to the formation of the active form. DNApol-γ, is inhibited by NRTI triphosphates (e.g. AZT-TP) to deplete mtDNA by competitive inhibition with the normal pool of nucleotide triphosphates. Adapted from reference 106.
Mitochondrial genome concatemers influence cellular and mitochondrial functions

The mtDNA encodes only 1% of the mitochondrial proteins; however, it is crucial for mitochondrial stability. The mtDNA-encoded genes are essential subunits for OXPHOS system assembly and function. Therefore, the mtDNA is necessary for cellular energy production in the form of ATP through OXPHOS from dietary metabolites (172).

Variations in the mtDNA are highly detrimental for cellular homeostasis (38, 115). Full and partial deletions of the mtDNA have been reported in several conditions including mitochondrial myopathies (69), Kearns-Sayre syndrome (104), PEO (214) and Pearson syndrome (154). More than 200 point mutations and rearrangements of the mtDNA have also been associated with human disease (29, 103), and some of them have been directly linked to tumorigenesis (18, 27). Penta JS et al. proposed that it is possible that the oxidatively stressed environment of tumors may result in high rates of mtDNA mutations and reduced expression of mtDNA-encoded polypeptides (142). Strikingly, mutated mtDNA in tumors has been found as mostly homoplasmic, meaning that all cells within a tumor carry the same mtDNA mutation, a phenomenon that suggests selective advantage to these mutations (138). Recently, it was reported that particular mtDNA mutations can contribute to tumor progression. Cells bearing a mutation in the mtDNA gene encoding
NADH dehydrogenase subunit 6 (ND6), have a defective respiratory complex I and subsequently overproduce ROS that increase metastatic potential (73). Nevertheless, dimerization or concatemerization of the mtDNA have not been related to any cellular disorder so far. This dissertation shows evidence that formation of mtDNA concatemers is associated with changes in mitochondrial function and with neoplastic transformation in lymphoid cells.

In this study, several significant alterations in mitochondrial functions and cellular factors associated with mitochondria in EL cells were identified. Probably the decrease in Fas-mediated apoptosis is most noteworthy. It has been shown that tumor cells often escape immune surveillance by down-regulating the Fas pathway or by using it to their advantage (91). For example, some tumors increase Fas ligand (FasL) expression to induce apoptosis of infiltrating lymphocytes (92). Also, some viruses can promote their propagation by expressing Fas-inhibitory proteins (11, 185). In regards to mtDNA, the cleavage and cytoplasmic release of cytochrome b (a mtDNA-encoded protein) has been directly linked to Fas-induced apoptosis (91). However, more studies are required to evaluate whether these changes in EL cells are a consequence of malignant transformation or represent the result of mtDNA duplication and mutation.
Increased intracellular ROS

The role that intracellular ROS play in cellular biology has been controversial. ROS are highly reactive due to the presence of unpaired valence shell electrons. Therefore, during environmental stress, ROS levels increase (presumably within the mM range) and cause random deleterious oxidation of macromolecules, a condition known as oxidative stress (102, 114). MtDNA is constantly subjected to oxidative damage due to direct exposure to ROS as direct byproducts of OXPHOS. This damage results in mutations in the mtDNA, which are associated with aging and various mitochondrial disorders (79). On the other hand, the treatment of human cells with hydrogen peroxide results in a transient increase in mtDNA copy number, suggesting that ROS can regulate mtDNA replication (207). Furthermore, the amount of mtDNA correlates with ROS levels in cell lines carrying different mouse mtDNA haplotypes (132).

Contrary to their role as inducers of cellular toxicity, several lines of evidence have shown that high ROS levels are associated with cell survival by deregulation of the PI3-kinase/Akt pathway during tumorigenesis (35, 52). Moreover, mitochondrial ROS could also contribute to neoplastic transformation, both as a tumor inducer by causing mutations in the nuclear DNA in proto-oncogenes and tumor-suppressor genes, and as a tumor promoter through promoting cellular proliferation (198). At low levels (within the
µM range), ROS have been found to be active mitogens. ROS are presumed to act through interaction with various kinases including Src kinase, protein kinase C, MAPK, and receptor tyrosine kinases, and with several transcription factors such as Fos, Jun and NF-κB (124). A significant reduction of glutathione levels was observed in EL cells. This may be associated with the elevation in ROS production, since glutathione works as a biological scavenger of ROS (64).

**Herpesvirus saimiri-immortalized human T-cells contain mitochondrial genome concatemers**

In order to determine if cells undergoing transformation were able to produce concatemers of mtDNA similar to the ones observed in the primary AIDS-associated lymphomas and lymphoma cell lines studied, immortalization of normal T-cells by infection with Herpesvirus saimiri was performed. This experimental approach was chosen because it has been successfully used in the past to obtain human, rabbit and marmoset transformed T-cell lines (126, 127, 181).

Discrete bands of mtDNA concatemers were detected by Southern blotting in the T-cell lines T-484 and BH36, immortalized with H.saimiri.
Additionally, a fainter band representing a small amount of concatemers was also detected in normal T-cells. This indicates that the process of formation of concatemers might also take place under normal conditions; however, resolution of concatemers into monomers might be defective in neoplastically transformed cells resulting in accumulation of concatemers.

Statistical analysis of relative densitometric units shows that the mean values between concatemer and monomer amounts for each sample is significant (P-values<0.005). This means that there may be an intrinsic mechanism that keeps total mtDNA amounts (and possibly concatemer/monomer ratios) relatively constant within these cells. Notably, the higher amount of concatemers in immortalized T-cells was also statistically significant (P-value<0.002) in comparison to the much lower levels detected in normal T-cells. This implies that formation and maintenance of mtDNA concatemers is a condition associated with immortalization of T-cells. However, further experiments would be necessary to determine whether mtDNA concatemers can promote oncogenic transformation of normal cells, or if they result as byproducts of cellular growth and proliferation observed in cancer.
Proposed mechanism of neoplastic transformation of lymphoid cells by formation and maintenance of mtDNA concatemers

Cellular homeostasis and energy production rely heavily on mtDNA stability (177). Mutations in the mtDNA have long been linked to the development and poor prognosis of various types of cancers (14, 27, 55, 73, 138). The physiological significance of mtDNA mutations in cancer cells was demonstrated by introducing a known pathogenic mtDNA mutation into the prostate cancer cell line PC3 via transmitochondrial cybrid fusions (143). Injection of these mutant and wild-type PC3 cybrids into nude mice gave a dramatic result. The PC3 cells containing the normal base (T8993T) barely grew at all. By contrast, the PC3 cybrids with the mutant base (T8993G) generated rapidly growing tumors that killed the mice (143). Staining the cellular nodules from both conditions revealed that the mutant tumors produced much more ROS than the wild-type nodules. Thus, underscoring the generation of increased ROS, associated with mutant mtDNA, in the pathophysiology of cancer (143).

Neoplastically transformed cells, however, can also harbor non-mutated mtDNA (198). Therefore, the presence of mitochondrial genome concatemers may represent an alternative mechanism of increasing intracellular ROS levels, as detected in EL cells. Recently, Lan Q. et al showed that increased mtDNA copy number was associated with a higher risk of developing non-Hodking’s
lymphoma (NHL) by analyzing blood samples from 208 NHL and healthy
individuals (97). This dissertation shows evidence that, independent of
configuration, overall mtDNA levels are increased in AIDS-associated
lymphomas, in lymphoma cell lines and in immortalized T-cells. Formation of
mtDNA concatemers is also associated with higher mtDNA levels observed in
these samples. Furthermore, imbalance and lower coupling efficiency of the
OXPHOS system have been reported in cells with abnormal mtDNA content
(67, 211). Defects in OXPHOS have long been known as causative of
increased ROS levels (49, 56, 87, 102). The diffusion of ROS into the nucleus
can then mutate proto-oncogenes, converting them into functional oncogenes.
Increased cytosolic and nuclear ROS activate a variety of cellular signal
transduction factors including NFκB, APE-1, Fos, Jun, and tyrosine kinases,
which drive the cell into replication. Consequently, variations in the mtDNA
(content and/or mutations) capable of affecting the normal function of the
OXPHOS machinery, result in chronically increased mitochondrial ROS
production, which can act as both a tumor initiator (mutation of proto-
oncogenes) and tumor promoter (activation of transcription and replication) (87,
176, 197) (Figure 24).
Cells containing mitochondrial genome concatamers show increased mtDNA levels (Figure 19) and higher ROS production (Figure 15). Neoplastically transformed cells upregulate mtDNA gene expression (190) generally resulting in a defective (uncoupled) OXPHOS system (14, 73), which can also produce higher ROS levels. ROS can inhibit tumor suppressor factors (p16ink4a, p53) and induce transcription factor activation (AP-1, NF-B, APE), which results in cellular proliferation (176, 198).
Finally, these experiments have established a clear correlation between mtDNA concatemerization and malignancy of lymphoid cells. However, it is not clear whether concatemerization is a contributing factor to oncogenesis, or alternatively, a result of continuing growth of immortalized cells. Resolution of these possible alternatives requires further investigation.

Summary and future directions

The development of techniques to study cccDNAs has been of importance in the understanding of the etiology of human diseases. This dissertation describes an experimental approach developed in our laboratory, and by which a novel extrachromosomal cccDNA was identified in AIDS-related lymphomas and lymphoma cell lines. This cccDNA is conformed by concatemers of the mitochondrial genome. Moreover, by immortalizing normal human lymphocytes with Herpesvirus saimiri, it was demonstrated that formation of mitochondrial genome concatemers is associated with oncogenic transformation of lymphoid cells.

In order to determine the relationship between formation of mtDNA concatemers and oncogenic transformation, two strategies are proposed. First, the fabrication and study of transplacental mitochondrial cybrids using cells containing
either wild-type or concatemeric mtDNA, with similar nuclear backgrounds, will
determine whether the presence of concatemers is sufficient to induce
neoplastic transformation. And second, the study of T-cells immortalized with
Herpesvirus saimiri could be useful to demonstrate if mtDNA concatemers are
simply byproducts of cellular immortalization. Additionally, an increasing time-
point assay after viral infection is proposed to determine when
concatemerization of the mtDNA takes place in these cells. Further studies on
the mechanism of formation and biological significance of mtDNA concatemers
may provide a new avenue in the chemotherapeutic treatment of lymphoma.
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Felipe Bedoya completed his undergraduate studies at Universidad de Los Andes in Bogotá, Colombia with a Bachelors of Science degree in Chemical Engineering. In 2003 he entered the University of South Florida (USF) as a Master’s student in the College of Medicine’s Bioinformatics and Computational Biology Program. In 2004, he started as a doctorate student in the Medical Microbiology and Immunology Program at USF (currently the Molecular Medicine Program). During his matriculation he was awarded with the Diversity Student Success Predoctoral Fellowship, the Academic Excellence Award and Graduate Educational Opportunity Grant. He is a member of the The Honor Society of Phi Kappa Phi and The National Scholars Honor Society. He has published two manuscripts with first-authorship, one in the *Journal of Immunology* (2007) and another in *Leukemia Research* (2009), which is based on his doctoral research. He was accepted to start his post-doctoral training in immunology at The Wistar Institute in Philadelphia, PA.