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FROM THE ARCHIVES

A Personal Scientific Odyssey

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The Early Days

When I was just a little kid growing up in Philadelphia, I had heroes just like any other kid. But my real hero was old Albert Einstein himself. I liked him even more than my basketball heroes on the Philadelphia Warriors - Paul Arizin, Tom Gola and of course Wilt Chamberlin. I had taken up basketball in Elementary School mainly to avoid having to pay the tough neighborhood guys bribes to take the trolley. After I somehow got accepted to the all-academic Central High and left the tough kids behind, I amazingly became really good at it. My afternoons and weekends were spent in fierce pick-up games at the local playground. In winter, we actually used to shovel the snow off the ground to play ball. I developed a shot known around the neighborhood as the "Pretzel Shot" and somehow made the Central High Varsity team. The high point in my career (and my life!) came when the Philadelphia Inquirer for some reason took a picture of one of my unsuccessful pretzel shots in a game against Germantown High and put it on the front page of the local section (Supplementary Fig. S1). It was all downhill after that for my basketball career.

But I never lost my hero worship of Albert Einstein and early on decided to become a scientist. And when I read George Gamow's fantastic book, "One, Two, Three Infinity", I was hooked for life. I did not know what field of science; I just knew that it had to be a field where I learned the meaning of everything. But alas, reality intruded and, after having a great Biology teacher at Central, Dr. Sam Lepow (Supplementary Fig. S2), I realized that even I could do Biology and that it was really interesting. Central turned out to be a formative life experience and was definitely the toughest school I ever went to, including College. I graduated older and a little wiser, and with a flat top haircut (Supplementary Fig. S3).

Princeton University and Oak Ridge National Laboratory

In my last year I somehow had the audacity to apply to some really top Universities, thinking that after I was rejected I probably could go to our local subway college, Temple U. But Princeton actually accepted me, with a fellowship, the chance to clean tables in the dining halls and a slew of student loans (that I was still paying off in my middle age). So off I went to Princeton. Princeton was great, but I never joined an Eating Club (Princeton's high class fraternities) and had to eat and recreate in the University alternative to Eating Clubs - Woodrow Wilson Lodge. Princeton was at that time all male and I sometimes felt like a monk in a cave.

My greatest failure at Princeton was to not make the basketball team. This was somewhat decided by my breaking and dislocating my right wrist in the summer while making one of my "pretzel shots" at my old Philly Playground, when I should have been selling Good Humor Ice Cream in my truck (Supplementary Fig. S4). But I did make the shot!.

Back at Princeton, after a flirtation with Philosophy, I went back to my early love, Biology, as my major. This was solidified by spending two summers working in the Biology Division at Oak Ridge National Laboratories, where I did thesis research under Hal Blum at Princeton and his friend at Oak Ridge, John Kirby-Smith. My project was to use Electron Spin Resonance to study Photodynamic

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Dyes. I wound up with blue and purple stained hands, a Nature paper (Simpson et al. 1963) and a real appreciation for this National Laboratory - but was really disappointed that the Biology Division was outside the security fence and I never learned any neat national atomic secrets. In addition to Kirby-Smith, I gained another science hero - the biophysicist, Dick Setlow. Dick was brilliant and yet down to earth. I went hiking with him on weekends in the Cumberland Mountains whenever I could. I wanted to go for a PhD but was not yet sure what field or graduate school would accept me. So I applied to several top schools that had many different fields, including one school recommended by my Princeton advisor, Hal Blum - Rockefeller University. All except Rock Tech required the Graduate Record Exam, and when I woke up the day of the exam at 10 AM and it had started at 9, I decided to go to Rockefeller! I had a brief interview with the President, Detlev Bronk, and he accepted me on the spot - mainly on the word of his friend, Hal Blum. So it was off to New York.

Rockefeller University and Brazil

Rockefeller was an interesting and novel graduate school that had few if any formal courses and seemed to allow students to learn by osmosis in the weekly dinners and luncheons with famous scientists. In any case, after a year learning by osmosis, I found an announcement on a bulletin board from an Institute in Brazil in the Amazon that was looking for American researchers. I had always dreamed of hanging from trees in the jungle like Tarzan and immediately applied. I was accepted and actually was asked what I would like to do research on! I did not let my limited scientific knowledge from interfering with my adventure in the jungle, so I asked people around Rockefeller what I could do. The suggestions ranged from collecting fern spores to collecting *Drosophila* sibling species, and finally Dr. William Trager, a famous parasitologist at Rockefeller, suggested that I work with parasites and look for the forest reservoir of mucocutaneous leishmaniasis, a disfiguring disease caused by *Leishmania braziliensis*. In fact he had an Assistant Professor in his lab, Dr. George Jackson, a nematologist, who was going to Rio de Janeiro in September for a malaria meeting and would be happy to accompany me and save my reputation while teaching me about parasites. So I got a textbook on parasitology and flew down to Manaus, 1,000 miles up the Amazon, where the National Institute of Amazon Research or INPA was located. I read the chapters on protozoan parasites and was up to the chapters on worm parasites when the plane arrived in Brazil. That is why to this day I know very little about worm parasites! George could only come down a few weeks later so I had to brazen it out somehow. They called me “Dr. Larry” probably because I wore a tie, and I did not discourage them. They gave us a lab in the Institute in Manaus and a person who could trap animals for us in the forest. My hunter’s name was Mozart Mello and he had two assistants and a jeep and driver to take us to some primary rain forest within 70 km from the city (Supplementary Fig. S5). Wow! I must have been the only guy from Philadelphia who had a hunter and two assistant hunters!! When they asked what I wanted to do, I of course said “field work” until George arrived, so off to the forest we went. George arrived in a few weeks and the real work began. We (i.e. Mozart) trapped animals and took them back to the lab and examined them for parasites, especially blood borne parasites, in an ultimately futile search for the forest reservoir of *L. braziliensis*.

This summer, however, started my love affair with Brazil and opened my eyes to the beauty and fascination of parasites. I decided to ask Bill Trager when we returned to New York if I could do my PhD thesis with him on *Leishmania*. Trager suggested I work on an unusual organelle that contained DNA but about which very little was known - the kinetoplast. At that time all we knew was that the *Leishmania* mitochondrion contained a large compact mass of DNA known as kinetoplast DNA that stained well with certain dyes and appeared in the EM as a fibrillar DNA structure. Our favorite model system was and still is the nonpathogenic trypanosome, *Leishmania tarentolae,* which was originally a parasite of a gecko, since Trager had succeeded in developing a completely synthetic axenic culture medium for this parasite (Trager 1957). My thesis research involved two separate projects: The effect of acriflavin on the kinetoplast of *L. tarentolae* (Simpson 1968b) and the differentiation of *Leishmania donovani* from the amastigote stage to the promastigote stage (Simpson 1968c). The latter project had the added flavor in that the parasite was infectious to humans, which kept me alert during the 30 hr long experiments.

After receiving my PhD from Rockefeller University, I went to Brussels for a postdoctoral year with Maurice Steinert, a leading researcher on kinetoplast DNA. During this year I learned how to isolate the kDNA as a single giant network and visualize it by light microscopy. Maurice also showed me how to collect eatable mushrooms from the Floret de Soignes, which his wife, Gilbert, would cook up for Sunday lunch.

UCLA and Kinetoplast DNA

I arrived at UCLA as an Assistant Professor in 1969, bright-eyed and bushy-tailed (Supplementary Figs. S6, S7), and my first graduate student, Agda da Silva, and I began to analyze kinetoplast DNA from an insect parasite, *Crithidia fasciculata,* and from *L. tarentolae*. We and others soon showed that this DNA consists of thousands (5000-10,000) of small circular DNA molecules known as minicircles, all linked together by catenation like rings in a chain, forming a giant network of DNA, and a smaller number (20-50) of large "edge loops" which we now attribute to maxicircles (Simpson and da Silva 1971) (Fig. 1A,B). We found that we could easily isolate this DNA due to its high sedimentation value and visualize it in the light microscope as I had learned in the Steinert lab. The genetic role of the minicircles was entirely mysterious at that time, but nevertheless the kDNA network was so bizarre that we decided to study the replication and segregation of minicircles.

Replication of Kinetoplast DNA

The study of replication of minicircles occupied my early years at UCLA. We showed that *L. tarentolae* could be synchronized in culture with hydroxyurea and that the kDNA S phase was fairly synchronous with the nuclear DNA S phase (Fig. 2A) (Simpson and Braly 1970). We also developed a method to isolate the single mitochondrion of *L. tarentolae* (Braly et al. 1974). This method was based on an observation I made during my thesis research that the parasites would swell in hypotonic buffer and that their single mitochondrion would also swell and would shrink again if isotonic conditions were restored (Fig. 2B) (Simpson 1968a). We also found that a mild shear force would separate the mitochondrial vesicle from the cell ghost and flagellum. Finally equilibrium centrifugation in a Renografin density gradient yielded a very clean mitochondrial fraction. This basic method is still used today. An alternative method using a Percoll gradient was used when we wanted to obtain enzymatically active mitochondria for some purposes.

When cloning and restriction enzymes became available in the 1970's, we applied these techniques to the study of the *L. tarentolae* minicircles. The restriction patterns were highly complex, although all of the circles were the same size, and these patterns actually changed somewhat after several years' culture in the lab (Simpson et al. 1980). We sequenced several minicircles and found that they were organized into a single conserved region and a single variable region. The conserved regions were found by the Englund (Ntambi and Englund 1985) and Ray (Birkenmeyer and Ray 1986; Ray 1989) labs to contain the origins of replication of the two strands of DNA.

We had previously discovered that the large size of the networks lent itself to studying kDNA repli-

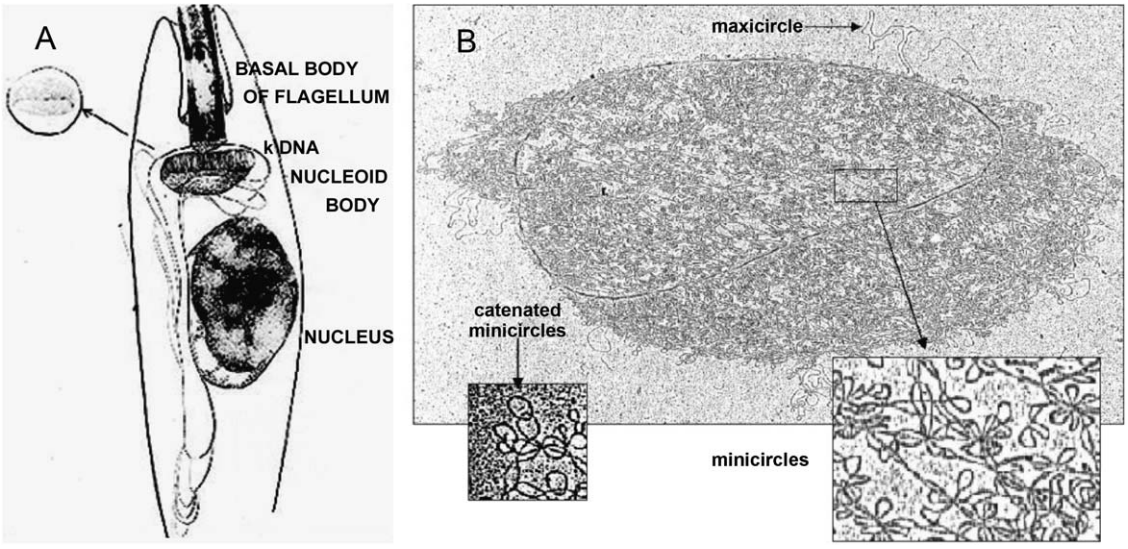

Figure 1. Kinetoplast DNA. **A.** 3D reconstruction of the single mitochondrion of *L. tarentolae (*by Frank Kretzer, UCLA) showing the kDNA nucleoid body in the matrix adjacent to the basal body. **B.** *C. fasciculata* kDNA network spread on grid and shadowed with platinum-palladium. Maxicircle edge loops are shown extending from the network. Inserts show the catenated minicircles.

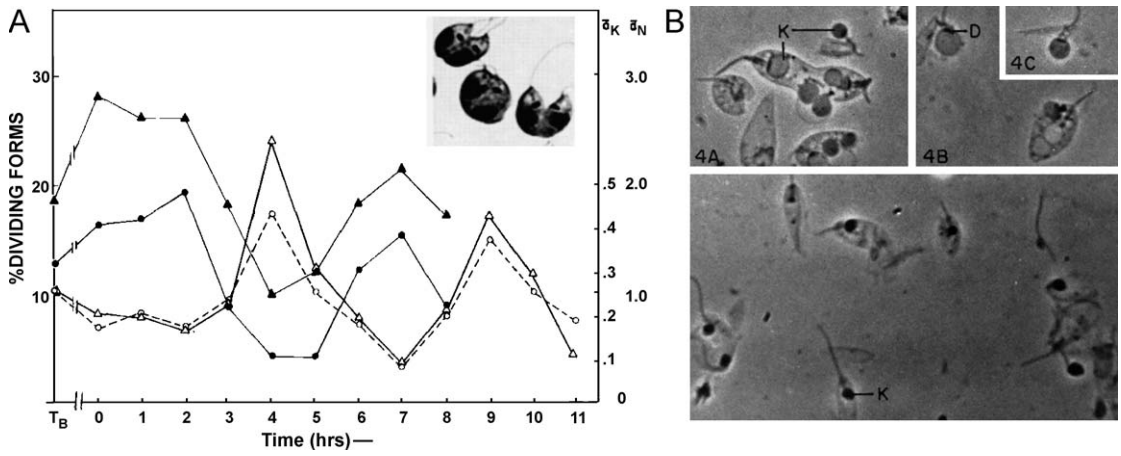

Figure 2. A. Hydroxyurea-synchronized *L. tarentolae* showing synchronization of kinetoplast DNA and nuclear DNA synthesis. Cells treated with 200 μg/ml HU for 10 hr. and then washed. kDNA and nDNA synthesis measured by autoradiography. Symbols: △ = 2K-1N + 2K-2N; ◯ = 1K-2N + 2K-2N; ▲ = average number of silver grains per nucleus in 15 min pulse with ^{3}H thymidine; ● = average number of silver grains per kinetoplast in 15 min pulse. Insert: representative synchronized dividing cells. **B.** Upper panels: *L. tarentolae* cells treated with hypotonic Tris-EDTA, phase contrast. The swollen vesicles (labeled "K") usually attached to the flagellum probably represent the entire single mitochondrion (or at least the kinetoplast region) with the kDNA seen as a dense strip (labeled "D"). Lower panel: Lysed cells resuspended in 0.25 M sucrose, resulting in osmotic shrinking of the kinetoplast-mitochondrion (K). From J Protozool **17**: 511-517 (1970), J Protozool (1968) **15** 132-136, with permission.

cation. We showed that a short pulse label with H^3 thymidine led to two antipodal nodes (Simpson and Simpson 1976) (Fig. 3A). A chase with unlabeled thymidine led to an annular ring of grains that progressed towards the central region (Fig. 3B) (Simpson et al. 1974). We speculated that there was some type of mobility of minicircles within the network, but this was not really understood until the elegant work of Englund beginning in the 1980's (Morris et al. 2001).

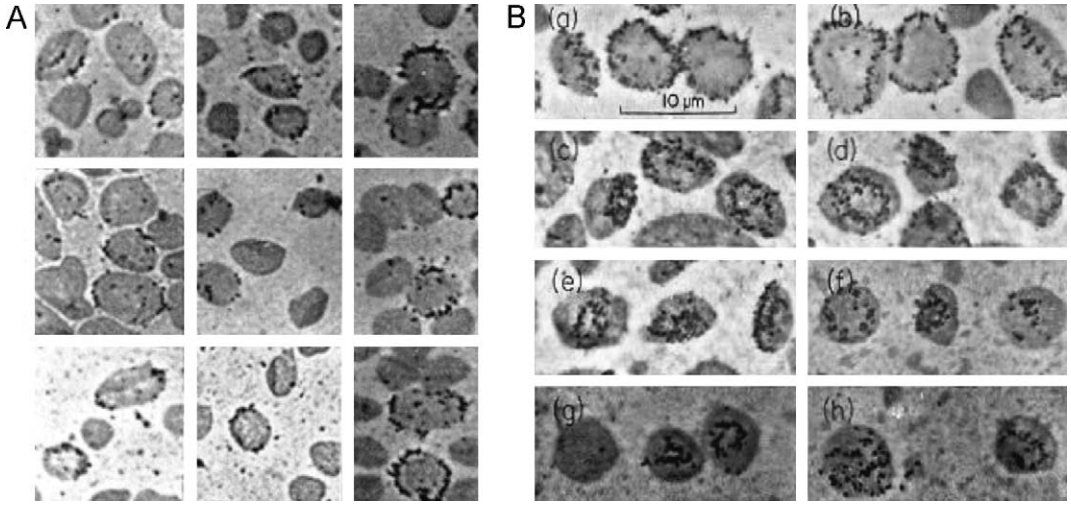

Figure 3. A. Autoradiographs of kDNA networks isolated from *C. fasciculata* cells pulse-labeled with ^{3}H thymidine for 30 sec. (upper) to 10 min. (lower). **B.** Same as (A) but cells were pulse-labeled for 10 min (a,b) and chased with unlabeled thymidine for increasing periods (c-h). From J Protozool (1976) **23:** 583-597, Biochim Biophys Acta (1974) **349**: 161-172, with permission.

Maxicircle Kinetoplast DNA

Around that time - 1973 - a graduate student, Ron Wesley, and I were investigating the thousands of enigmatic minicircles in the kDNA network to try to learn their genetic function. Renaturation kinetic or "CoT" curves were run of sonicated kDNA to see if the minicircles were homogeneous or heterogeneous. To our surprise there were two components to the renaturation curve, the majority of the DNA renatured fairly rapidly but a minor portion ($\sim$ 5%) renatured significantly slower, suggesting the presence of a minor higher complexity component in addition to minicircles (Wesley and Simpson 1973).

Around the same time, Kleisen and Borst in Amsterdam discovered the presence of a minor higher complexity DNA species in the kDNA network by direct gel analysis after digestion with restriction enzymes, and they baptized it the "maxicircle" DNA (Kleisen and Borst 1975; Kleisen et al. 1976). This clearly was the DNA responsible for our slowly renaturing component. They calculated that there were around 10-20,000 catenated minicircles and around 20 maxicircles, which appeared in the EM of kDNA networks as "edge loops".

In 1979, we found that maxicircle DNA cut with a single site enzyme showed a lower buoyant density in a dye-CsCl equilibrium gradient than the bulk of the minicircles released from the network due to the maxicircle's higher A + T content. This led to a simple method to isolate the maxicircle DNA (Simpson 1979).

Minicircle DNA of *Trypanosoma cruzi* - Schizodeme Analysis

In 1977, Carlos Morel, a young Brazilian scientist then at the University of Brasilia, published a paper which showed that strains of *T. cruzi,* the causal agent of Chagas disease, could be distinguished by restriction enzyme gel profiles of kDNA. I found this of interest and invited him to come to my lab. In 1979, after finally having obtained a travel permit from the Brazilian military government and a grant from CNPq/NSF, Carlos arrived carrying a slew of *T. cruzi* cell lines from patients in liquid nitrogen. Morel was a gifted investigator and soon confirmed and extended his results by using additional enzymes and high resolution gels. The gel profiles were highly complex and were indeed characteristic of different strains of the parasite (Fig. 4A). This classification method proved to be more sensitive than the classical "zymodeme" enzyme analysis. We coined the term, "schizodeme", to indicate groups

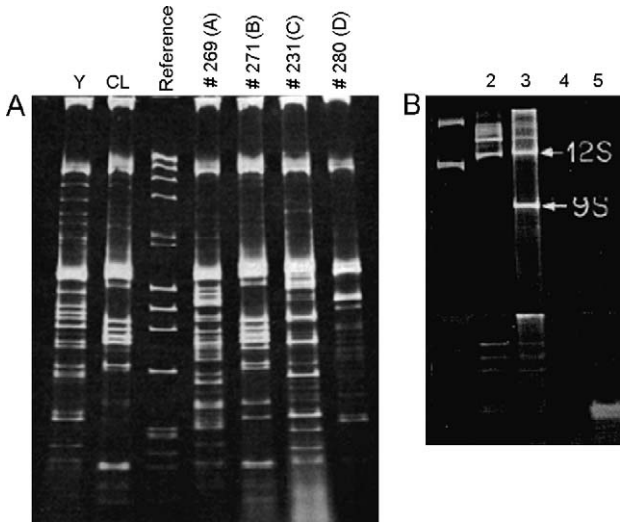

Figure 4. A. Acrylamide gel profile of Hinf I - digested kDNA from the Y and CL lab strains of *T. cruzi* and from 4 strains which represent zymodemes 1-4 isolated from Chagasic patients. **B.** Acrylamide gel (4-10%) of cytosolic RNA (lane 2) and kRNA (lane 3) from *L. tarentolae*. *E. coli* rRNA (lane 1), 4S (lane 4) and 5S RNA (lane 5) are shown as markers. From Proc Natl Acad Sci USA (1980) **77** 6810-6811, Cell (1978) **14** 169-179, with permission.

of *T. cruzi* that showed similar kDNA restriction profiles and schizodeme analysis of kDNA became a standard method to classify strains of *T. cruzi* (Morel et al. 1980). So, in spite of our lack of knowledge of the genetic role of minicircles, they could be used to classify strains of *T. cruzi*! I would call this "utility without knowledge".

One day around 1988 I received a phone call from a friend at Cal Tech who asked if I wanted to look for *T. cruzi* in ancient mummies from Chile. I told him that we had been doing PCR of *T. cruzi* minicircles and that may provide a method of detection, since there are thousands of minicircle targets per cell. He sent over some mummy tissue which looked like horsehair, and we proceeded to get nowhere quickly in isolating DNA from this material, which turned into a black goo when detergent was added. But it came to me that perhaps the minicircle PCR method could also be used to detect *T. cruzi* parasites in live Chagas Disease patients not just in mummies. My graduate student, Nancy Sturm and Wim Degrave, a visiting member of Morel's lab in Brazil, and later, Herb Avila, a graduate student, were instrumental in this work, and soon discovered that the minicircles in this species contain four conserved regions and four variable regions (Degrave et al. 1988). We decided to target the conserved

regions to detect all *T. cruzi* stains and the variable regions to do schizodeme classifications. They developed a method to isolate DNA from a large amount of blood and soon showed that they could diagnose the presence of *T. cruzi* in patients' blood at a high sensitivity and high specificity, using PCR of kDNA minicircles (Avila et al. 1993). This method is still being used in Chagas endemic countries for diagnosis and classification. Attendance at the annual Brazilian Chagas Disease Meetings at Caxambu became of course obligatory (a tough duty, but someone had to do it!), and a continual stream of students working on *T. cruzi* traveled between Morel's lab in Rio and my lab in Los Angeles.

The Maxicircle-Encoded 9S and 12S Mitochondrial Ribosomal RNAs

Work on *L. tarentolae* kDNA was, however, the major research thrust of my lab. Since we had developed a method to isolate a highly purified fraction of mitochondria from *L. tarentolae*, Agda Simpson decided to examine the question of transcription of the kDNA. There were two major RNAs visible in electrophoretic gels, which we called the 9S and 12S RNAs (Fig. 4B) (Simpson and Simpson 1978). Initially we thought that these were minicircle transcripts but soon learned that they were derived from maxicircle DNA (Simpson and Simpson 1978).

These RNAs turned out to be the mitochondrial ribosomal RNAs, in spite of their small size. Several students including Vidal de la Cruz, Mike Muhich and Nick Neckelmann started to analyze these RNAs. They found that the 9S RNA could be folded into a standard small rRNA mode, albeit with structural features missing (de la Cruz et al. 1985a). The 12S RNA was more difficult to fold, but several highly conserved regions were found to fit the general large rRNA model (de la Cruz et al. 1985b). Similar results were obtained for *C. fasciculata* (Sloof et al. 1985; White et al. 1986).

Work on the *Leishmania* 9S and 12S rRNAs has continued in the lab of Dmitri Maslov, a postdoctorate who is now a Professor at the University of California at Riverside. In 2009, in a real tour de force (pardon my French!), in collaboration with Raj Agrawal, an expert on cryo-EM of ribosomes, Maslov obtained a 3D reconstruction of the mitochondrial ribosome of *L. tarentolae* with both ribosomal RNAs precisely localized. (Sharma et al. 2009). This mitochondrial ribosome was more porous than other known ribosome structures and major portions of key functionally conserved sites, such as the channel where mRNA enters, the transfer RNA passage, and the region where nascent polypeptides exit, contain *Leishmania*-specific proteins, rather than familiar ones. These results really gave me a warm and fuzzy feeling!

Identification of Maxicircle Genes and the Missing Genes in *Trypanosoma brucei*

To return to the exciting years of the 1980's, we and others had shown that the maxicircle molecules, which range in size from 23,000 bp to 36,000 bp in different species, appeared to represent the informational DNA in the mitochondrion and contain several of the same genes also found in other mitochondrial DNA molecules: the large and small mitochondrial ribosomal RNAs (9S and 12S RNAs), three subunits of cytochrome oxidase, apocytochrome b, four subunits of NADH dehydrogenase. There were also several proteins with still unknown functions. All of the identified structural genes are involved with electron transport in the inner membrane of the organelle, as in human cells. I will never forget the initial discovery of the apocytochrome b gene by my graduate students, Vidal de la Cruz, Nick Neckelmann and myself. We had a sequence of the pLt120 maxicircle fragment and ran hydropathy plots of all six reading frames (de la Cruz et al. 1984). The dot matrix printout was stretched out on the hallway of the Life Science Building in front of my lab, and we walked up and down the hall trying to recognize by eye the characteristic hydropathy profiles of known mitochondrial genes. It was very exciting when we saw a short stretch that fit the Cyb pattern, and all the other genes rapidly fell in place. Clearly this was very different from the sophisticated bioinformatics methods used today for gene identification, but this was 1983!.

There were several early warning signs that something was unusual about this mitochondrial genome: two of the genes (COII, ND7) apparently had an extra or a missing nucleotide which created a reading frame shift which would terminate translation if not corrected. These -1 or +1 frame shifts turned out to be present at the same relative locations in the genes from different genera which we knew were separated by at least 100 million years of evolution. Another problem was that several of the genes lacked AUG methionine codons for initiation of translation. And, finally, in a collaboration with Ken Stuart in the Seattle Biomedical Research Institute, who had done the *T. brucei* maxicircle sequence, we showed by dot matrix analysis that,

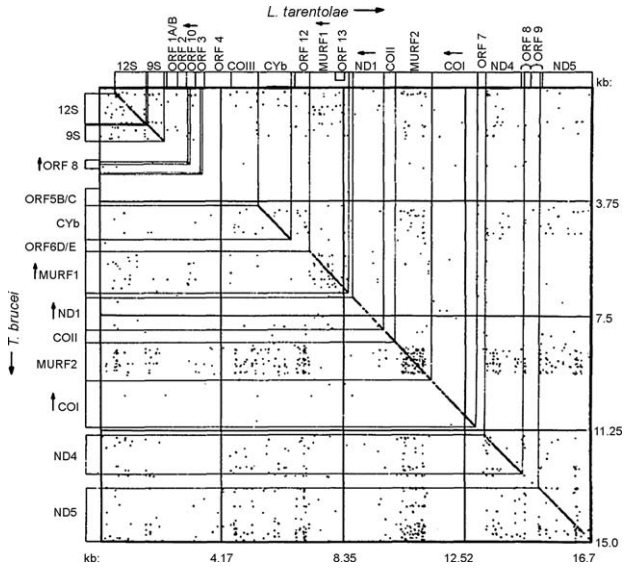

Figure 5. A dot matrix comparison of informational portion of maxicircle genomes from *L. tarentolae* and *T. brucei*. Window of 31 amino acids and stringency of 11. From J Biol Chem (1987) **262**: 6182-6196, with permission.

although the relative localizations of genes in the maxicircle genomes of *L. tarentolae* and *T. brucei* were very similar, three genes present in *L. tarentolae* were missing in the maxicircle genome of *T. brucei* (ND7, COIII and MURF4) (Simpson et al. 1987). These genes were substituted in *T. brucei* by shorter sequences that were relatively rich in G's (Fig. 5). Short non-conserved G-rich sequences were also present between genes.

U-insertion/deletion RNA Editing

The problem was solved and a Pandora's box of additional problems was opened up in 1986 when Rob Benne's laboratory at the University of Amsterdam published a sequence of the COII mRNA and found 4 extra U's in the conserved frameshift region that were not encoded in the DNA (Benne et al. 1986)!. The presence of these 4 U's inserted at 3 sites neatly overcame the -1 reading frameshift and allowed the mRNA to be translated.

My graduate student, Janet Shaw, in collaboration with Ken Stuart and his postdoctorate, Jean Feagin, soon confirmed this by sequencing both the maxicircle DNA and the mRNA from two species, and Janet also found several other more dramatic examples of this phenomenon that Benne had termed "RNA editing". For example, the Cyb mRNA was edited within the 5′ end by the insertion of 39 U's at 15 sites, thereby creating 20 new amino acids at the amino end of the protein, including an AuG translation initiation codon (Feagin et al. 1988b).

Deletions of U's were also found to occur in some genes, such as the COIII gene of *L. tarentolae,* although at a lower frequency (Fig. 6) (Shaw et al. 1988). We coined the word "cryptogene" (hidden gene) to describe genes whose transcripts are edited within coding regions, and we called that region of the mRNA which is to be edited the "pre-edited region".

And then one day in 1988, I got a call from Jean Feagin who told me that she had found the missing COIII gene in *T. brucei.* It was actually there all the time but was a truly hidden cryptogene since the transcript was so extensively edited with hundreds of U additions over almost the entire length that the mature edited mRNA was nearly twice the size of the gene (Feagin et al. 1988a). The edited *T. brucei* COIII sequence made the cover of Cell and suddenly trypanosome RNA editing became the "talk of the town". These were heady times. We could now go to meetings and not be relegated to the last talk on the last day, after the buses had left, with the introduction "and now for something really different". The Stuart lab soon found that this was the case also for the other two missing genes in *T. brucei* (Bhat et al. 1990; Koslowsky et al. 1990). Janet Shaw and I decided to call this extensive type

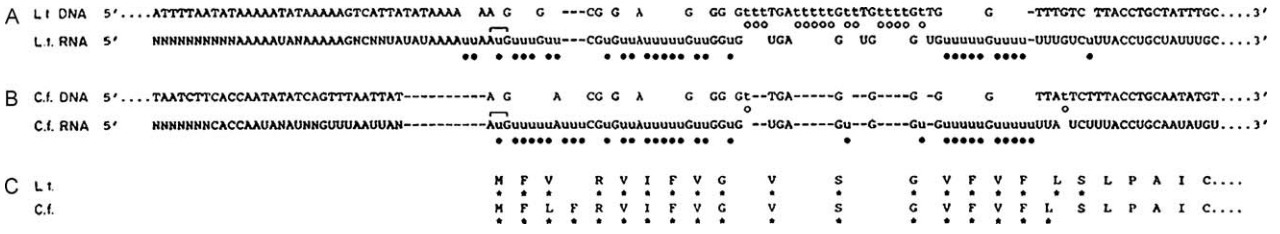

Figure 6. Editing of *Leishmania tarentolae* and *Crithidia fasciculata* COIII mRNAs. **A.** Lt maxicircle COIII DNA and edited COIII mRNA sequences aligned. The inserted u's are indicated by black dots. The deleted u's (or T's) indicated by open dots. **B.** Same as (A) for *Cf* DNA and RNA. **C.** Translated amino acids from edited mRNAs with conserved residues indicated by *. From Cell (1988) **53**: 401-411, with permission.

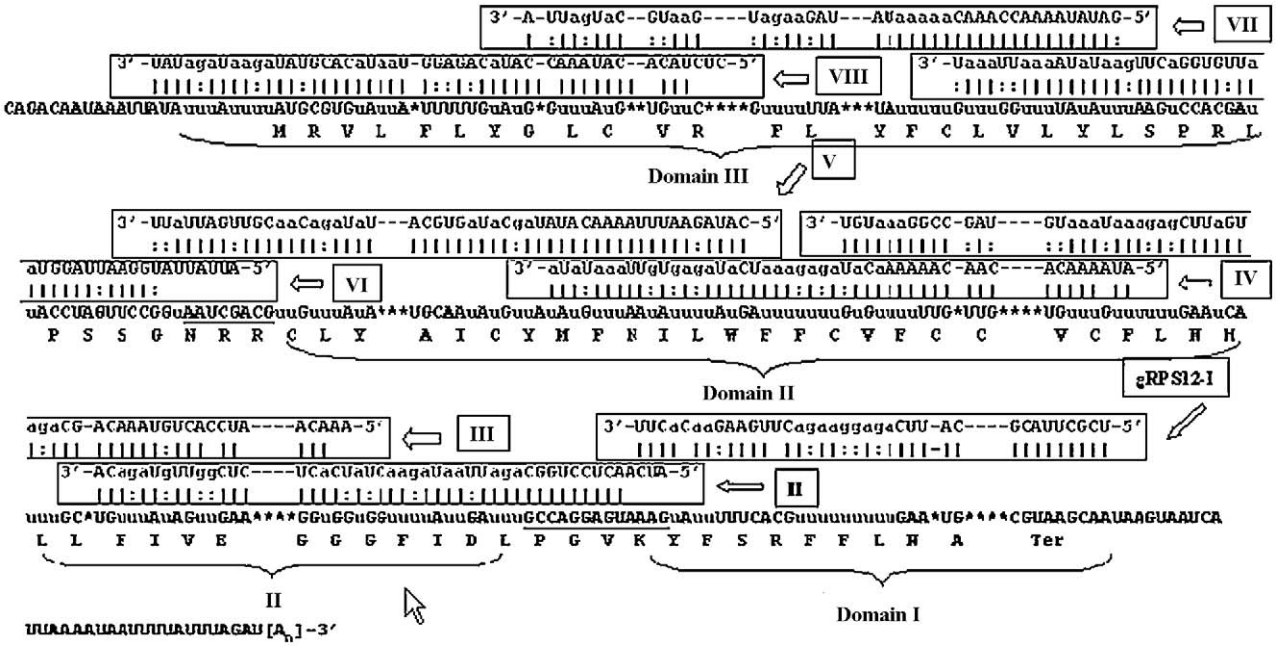

Figure 7. Sequences of edited *Lt* RPS12 mRNA and overlapping cognate gRNAs. There are 3 separate domains, each edited separately. gRNAs are indicated and boxed. Inserted residues are shown as "u" and deleted residues as *.

of editing "pan-editing" (Simpson and Shaw 1989). Dmitri Maslov soon showed that a G-rich region in the *L. tarentolae* maxicircle actually encoded ribosomal protein S12 which provided another example of pan-editing (Fig. 7) (Maslov et al. 1992). We were now able to construct a map of the entire coding region of the *L. tarentolae* maxicircle.

3′-5′ Polarity of Editing and Misediting

An important insight into the mechanism of RNA editing came when the Stuart lab found that the pan-editing of the COIII mRNA of *T. brucei* appeared to occur in an overall 3′ to 5′ direction (Abraham et al. 1988; Feagin et al. 1988a). This suggested that the process occurred after transcription, since transcription goes 5′ to 3′. Nancy Sturm found the same phenomenon to occur in the 5′-edited genes in *L. tarentolae*. To study this, Nancy decided to PCR amplify partially edited mRNAs. I told Nancy that she was cheaper than a PCR machine, which in any case I had no funds to purchase, so she performed the PCR reactions manually, adding the Taq polymerase before each cycle! Nancy sequenced over 400 partially edited mRNAs from the Cyb and COIII genes that were generated by PCR amplification from mitochondrial RNA and found that the region between the fully edited 3′ side and the unedited 5′ side of the mRNA, which we termed the "junction region", contained a variety of partially edited sequence patterns. In the case of the Cyb RNAs, almost all of these patterns could be arranged into a precise 3′ to 5′ progression of editing. However, in the case of COIII, only 58% of the patterns showed this precise polarity. The rest showed unexpected or "misedited" *editing* patterns, in which U's were added at sites not edited in the mature transcript or U's were added in the 5′ region before U's were added in the 3′ region (Sturm et al. 1992; Sturm and Simpson 1990b). Carolyn Decker and Barbara Sollner-Webb at Johns Hopkins Medical School analyzed partially edited mRNAs for Cyb and COIII from *T. brucei* and found a high percentage of unexpected patterns in the junction regions for both genes (Decker and Sollner-Webb 1990a). Several theories were suggested for the origin of these misedited sequences, including Nancy's "misediting by misguiding" theory (see below), and all turned out to be true in some cases.

Discovery of Guide RNAs

U-insertion/deletion RNA editing presented a challenge to the central dogma of genetic information transfer since there appeared to be no nucleic acid

template for this newly added sequence information. We had, however, not given up on the central dogma and two postdoctoral fellows in my laboratory, Beat Blum and Norbert Bakalara, decided to give it one last try. They performed a computer search of the known *L. tarentolae* maxicircle sequences for short DNA sequences that could give rise to RNAs with complementarity to either entire or portions of known edited RNA sequences. In addition to the classical Watson-Crick base pairs C-G and A-U, they decided to allow for G-U base pairs (Fig. 8A) since these are bonafide base pairs in rRNAs and tRNAs. This turned out to be the trick! Seven such short sequences for four of the known edited genes were immediately found scattered throughout the maxicircle between known genes. In a few days we had synthesized oligonucleotides for primer extension to verify the existence of the RNAs and within one very exciting week we had definitive evidence for the existence of small RNAs in the mitochondrion which were transcripts of these sequences. These RNAs also contained sequences at their 5′ end which could form duplex regions with the mRNAs just downstream of the pre-edited regions, which we termed the "anchor regions", since these provided an ideal way to anchor the short RNAs to the mRNAs by forming a double-stranded hybrid just downstream of the region that is to be edited. These small RNAs had an unusual mobility in gel electrophoresis; they migrated in the form of 20-30 bands each differing by a single nucleotide in size. Based on the previous use of the term, "internal guide sequences", in Group I self splicing introns (Davies et al. 1987), we called these molecules "guide RNAs" or "gRNAs" since they contained the sequence information for editing (Blum et al. 1990). The secret to the editing sequence information was simple base-pairing to short gRNAs. One gRNA was found to be *in cis* at the 3′ end of the COII mRNA.

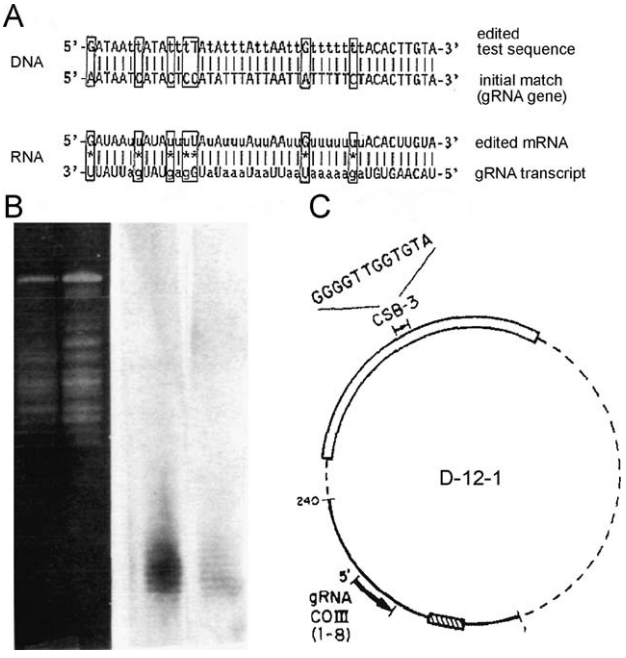

Figure 8. Discovery of guide RNAs. **A.** Upper: Alignment of edited *Lt* mRNA sequence versus *Lt* maxicircle DNA sequence. One of the best "hits" is shown. Mismatches are boxed. Lower: Alignment of edited *Lt* mRNA versus a putative gRNA transcribed from the *Lt* maxicircle DNA sequence in **(A).** Note that the mismatches in (A) are base pairs if G-U (*) is allowed. **B.** Lt kRNA run in acrylamide gel. Left lanes: ethidium bromide stained, showing tRNAs. Right lanes: Blots were probed with riboprobes from two cloned kDNA minicircles, showing gRNAs. **C.** The missing gRNA for COIII editing sites 1-8 is encoded in a partially sequenced minicircle within the "variable" region. The minicircle "conserved region" is boxed and the highly conserved CSB-3 sequence shown. From Nucleic Acids Res (1989) **17** 5427-5445, Cell (1990) **61**: 879-884, with permission.

Let me now digress from the historical development to return to the mid 1980's when we had just finished the sequence of the majority of the *L. tarentolae* maxicircle. One striking observation was that there were no obvious tRNA genes. The apparent absence of tRNA genes was really surprising since all mitochondrial genomes studied to that time contained tRNAs which are involved in mitochondrial protein synthesis. To examine whether some of the minicircle molecules or regions of the maxicircle which we had not yet sequenced encoded tRNAs, My graduate student, Homero Dewes and Agda Simpson hybridized labeled minicircle and maxicircle DNA to low molecular weight RNA from *L. tarentolae* mitochondria which was separated by electrophoresis in acrylamide. To our surprise, the minicircle probes lit up a cluster of 20-30 bands one nucleotide apart that migrated well ahead of the abundant mitochondrial tRNAs, indicating that

Base-pairing by guide-like RNAs *in trans* has since been found to explain the specificity of siRNA degradation of mRNA and the snoRNA-mediated specificity of methylation and pseudouridylation of rRNAs. And the *in cis*–guiding COII gRNA has provided a precedent for the specificity of A to I modification editing of mammalian mRNAs being determined by fold back of a downstream complementary sequence. But we still did not understand how the gRNAs could mediate the insertion and deletion of U's at precise sites.

these RNAs were even smaller than tRNAs; this RNA was such a minor component that we could not even see these bands by staining the gel! (Fig. 8B) (Simpson et al. 1989).

We now know that this was our first look at gRNAs which were present at low abundance in our kRNA preparations, but at the time we had no idea what these transcripts were. The heterogeneity of the gRNAs was soon found to be due to the presence of non-encoded 3′ oligo-[U] tails 15 - 30 nucleotides in length. And the fact that these appeared to be minicircle transcripts was also not appreciated at the time.

It was clear, however, that no mitochondrial tRNAs were encoded in maxicircle or minicircle DNA and therefore it seemed likely that tRNAs were imported into the trypanosome mitochondrion from the cytoplasm, as had been suggested several years before for *Tetrahymena* by my collaborator from the University of Pennsylvania, Yosh Suyama (Chiu et al. 1975; Suyama 1967). This was a heretical idea at the time, but an idea which is now generally accepted (Alfonzo and Soll 2009).

Guide RNAs are Encoded in Minicircles

At this time we had identified seven gRNA genes scattered all over the maxicircle with no positional relation to the cryptogenes for which they encoded information. These gRNAs had information for four of the five known cryptogenes, but we could not find a gRNA for the 5′ edited COIII gene in *L. tarentolae.* We had sequenced several kDNA minicircles by that time (Kidane et al. 1984), and Nancy Sturm decided to see if the misedited patterns she was analyzing could be encoded by gRNAs. To her surprise, she discovered the missing gRNA for COIII editing sites 1-8 encoded in a minicircle which we had partially sequenced! (Fig. 8C) (Sturm and Simpson 1990a). Both Nancy and I had completely forgotten our previous results that minicircles encoded short transcripts migrating in acrylamide as 15-30 bands one nucleotide apart but now with hindsight these results made sense.

Nancy's discovery was the first indication of a genetic function of the hitherto enigmatic minicircle DNA and explained neatly the previously observed sequence heterogeneity of minicircle DNA - each sequence class encoded a different gRNA within the variable region! Previous work by my graduate students, Getachew Kidane and Michael Muhich and further work by Nancy Sturm and Dmitri Maslov, resulted in the identification of a total of 17 different minicircle sequence classes of differing abundances in the *L. tarentolae* UC strain by cloning and sequencing.

Soon, minicircle-encoded gRNAs were also found in *T. brucei* and the closely related species, *T. equiperdum,* by the Hajduk (Pollard and Hajduk 1991) and Stuart (Shu and Stuart 1993) labs. There is one major interesting difference in that each minicircle in *T. brucei* encodes three different gRNAs rather than a single gRNA and the genes are located in the single variable region between three sets of 18 nt inverted repeats which are not present in *L. tarentolae.* Another difference is that previous workers had claimed that there were over three hundred different minicircle sequence classes in *T. brucei* versus the limited number found in *L. tarentolae,* suggesting that the total number of different gRNAs in *T. brucei* may be over 900. Some of these additional gRNAs are probably required for the three pan-edited genes that are only 5′-edited in *L. tarentolae,* and for the five pan-edited G-rich genes discussed below, but the function of the rest remains to be investigated. In 2003, we showed that the original estimate of minicircle sequence heterogeneity in *T. brucei* was an overestimate, but the number is still much larger than that found for *L. tarentolae* (Hong and Simpson 2003).

It was shown by Rob Benne that *Crithidia fasciculata,* a parasite of insects, contains the same maxicircle DNA-encoded gRNA genes as *L. tarentolae*, located at identical relative positions in the maxicircle genome (Van der Spek et al. 1991). The gRNA anchor sequences in *C. fasciculata* have several differences compared to the *L. tarentolae* sequences, but these are compensated for by mutations in the cryptogene sequences so as to preserve base-pairing. These results nicely confirm the role of gRNAs in editing by the same type of evolutionary argument that was previously used by Noller and Woese (Woese et al. 1983) to confirm the structure of double stranded regions in ribosomal RNAs.

G-rich Intergenic Maxicircle Sequences are Pan-Edited Cryptogenes

In our initial comparison of the mitochondrial genomes of *L. tarentolae* and *T. brucei,* we had noted that there were several stretches of sequences that were relatively rich in G residues (Simpson et al. 1987). Three of these in *T. brucei* were shown by the Stuart lab to be the three hidden pan-edited cryptogenes, ND7 (Koslowsky et

al. 1990) COIII (Feagin et al. 1988a) and MURF4 (Bhat et al. 1990) (now known as A6), but there were another six G-rich regions that were located between known genes in both species. Maslov, Sturm and Marian Peris showed that the transcript of G-rich region 6 in *L. tarentolae* is pan-edited by the addition of 117 U's at 49 sites and the deletion of 32 U's at 13 sites in three editing domains, producing an mRNA which encodes a protein for the small subunit of the mitochondrial ribosome (Fig. 7) (Maslov et al. 1992). It appeared that all six G-rich regions, perhaps in all kinetoplastid species, are pan-edited cryptogenes, encoding proteins important for the proper functioning of the mitochondrion. These additional cryptogenes would require approximately 50 additional overlapping gRNAs for mediating the required editing, which did not agree with the 17 different minicircle sequence classes found in the old *L. tarentolae* UC lab strain. In *T. brucei* there are only two gRNA genes in the maxicircle, but each minicircle encodes 3-4 gRNAs. It appears that in the African trypanosome there has been a movement of gRNA genes between the maxicircle and minicircle genomes. In fact the short inverted repeat sequences found adjacent to the gRNA genes in the minicircles of *T. brucei* could possibly represent the evolutionary remnants of transposition events in which gRNA genes moved from the maxicircle to the minicircle or between minicircles.

Loss of Editing in Old Laboratory Strain of *Leishmania tarentolae*

The 17 identified minicircle-encoded gRNAs in the old lab UC strain of *L. tarentolae* were clearly insufficient to account for the complete editing of all cryptogenes. This problem was solved by my graduate student, Otavio Thiemann, who is now a Professor in San Carlos, Brazil. He showed that the UC strain was defective in the editing of the G1-G5 cryptogene transcripts due to the absence of gRNAs for these editing events, whereas the recently isolated LEM 125 strain contained at least 32 additional gRNAs which encoded productive editing of the G1-G5 cryptogenes (Fig. 9) (Thiemann et al. 1994). The loss of gRNAs, accompanied by the loss of editing, was actually the first genetic evidence for the involvement of gRNAs in the editing process. We speculated that these minicircle-encoded gRNAs were lost during

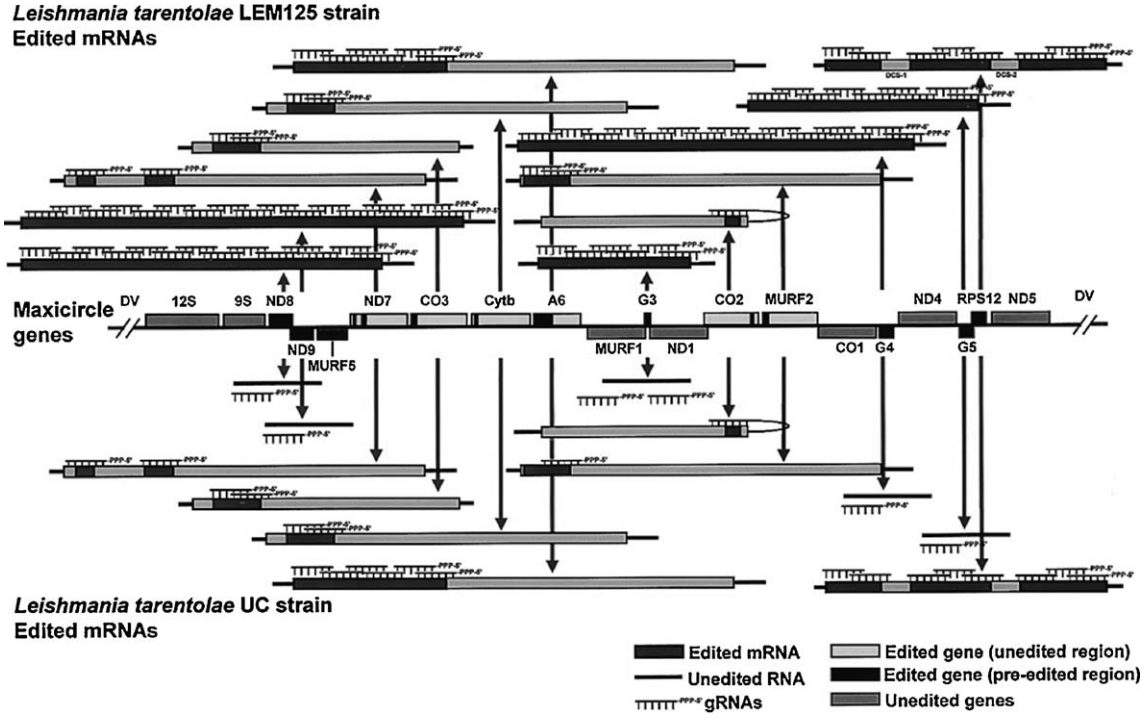

Figure 9. Diagrammatic comparison of gRNA-mediated editing of maxicircle cryptogenes in old lab UC strain of *L. tarentolae* and recently isolated LEM125 strain. The identified overlapping gRNAs that cause the 3′ to 5′ polarity of editing are indicated. All of the 80 predicted gRNAs in the LEM strain are shown although only 47 have been identified. From Proc Natl Acad Sci USA (2000) **97** 6986-6993, with permission.

the long culture history of the UC strain by loss of the specific minicircle classes. Nick Savill, a computer science collaborator, showed in fact by computer modeling with a few basic assumptions, that minicircle classes would fluctuate dramatically in copy number in the course of many generations in culture and that low frequency minicircle classes could be lost entirely in the absence of selection due to the random nature of the network minicircle segregation process during the cell cycle (Savill and Higgs 1999; Simpson et al. 2000).

Enzyme-Cascade Model for RNA Editing

Prior to the discovery of gRNAs, Norbert Bakalara, a postdoctorate in my lab, and Agda Simpson had identified an enzyme activity from purified mitochondria of *L. tarentolae* which could add U's to the 3′ terminus of any RNA molecule - a terminal uridylyl transferase or TUTase (Bakalara et al. 1989). This enzyme possibly was responsible for the addition of U's to the 3′ end of the gRNAs. They also showed the presence of a mitochondrial RNA ligase which could covalently link together two RNA molecules.

Armed with the knowledge of the existence of gRNAs and these enzymatic activities in the mitochondrion and of the 3′ to 5′ progression of editing on the mRNA, Beat Blum, Bakalara and I came up with a model for the role of gRNAs in RNA editing (Fig. 10) (Blum et al. 1990). We called this the 'enzyme cascade' model since it postulates a series of enzymatic reactions occuring in a multienzyme complex bound to the mRNA. We proposed that the initial interaction involves the formation of an anchor hybrid by a specific gRNA just 3′ of the pre-edited region on the mRNA. In addition to RNA/RNA interactions involved in the formation of an anchor, we believe protein factors which Agda Simpson had found to be complexed to the gRNAs assist in this initial specific interaction, perhaps by recognizing secondary structures formed by the mRNA itself or by the gRNA/mRNA hybrid. The next step was proposed to be a specific cleavage at the first mismatched base in the mRNA which liberates a free 3′ OH group. This cleaved mRNA fragment is a good substrate for the 3′ TUTase enzyme which could add one or more U's to the 3′ end. These added U's would then base pair with the guide A or G nucleotides in the gRNA, and then the two ends of the mRNA would be religated by the RNA ligase. This would result in a zippering up of the double helix in a 3′ to 5′ direction (on the mRNA),

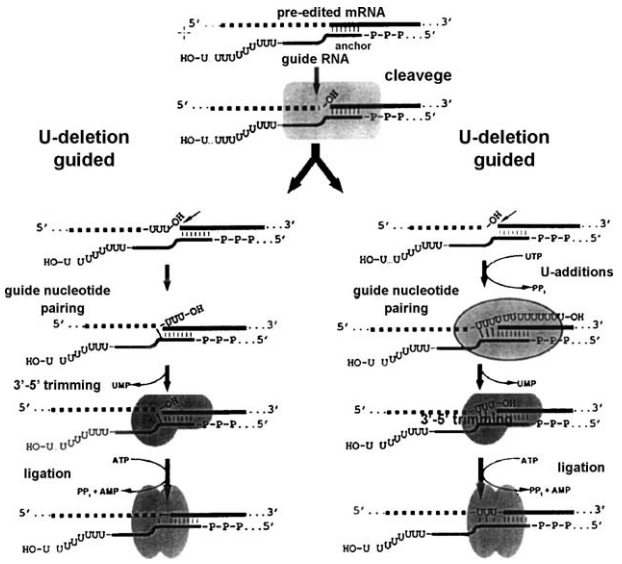

Figure 10. The original "enzyme cascade" model of RNA editing. U-deletion shown on left and U-insertion on right. Predicted enzymatic activities are indicated. We also suggested that the 3′ U-tail may base pair with the pre-edited sequence, thereby stabilizing the initial interaction. From Annal NY Acad Sci (1999) **870**: 190-205, with permission.

and the whole process would then reinitiate at the next mismatched base.

This model provided an explanation for the 3′ to 5′ polarity of pan-editing, as due to the creation by the downstream gRNA of edited mRNA sequence that was complementary to the anchor sequence of the adjacent upstream gRNA. This was shown clearly by Maslov for the A6 and RPS12 (Fig. 7) mRNA editing in *L. tarentolae* (Maslov and Simpson 1992). The model also explained the presence of unexpected editing patterns within the junction regions of partially edited mRNAs (Sturm and Simpson 1990b). Sturm and I had suggested that some of these patterns actually represent normal editing by inappropriate gRNAs or appropriate gRNAs in the wrong location or wrong reading frame, a process which we termed "misediting by misguiding", and which is enhanced by the presence of 'wobble' G-U and perhaps A-C base pairs. The formation of an anchor hybrid by the incorrect gRNA or the formation of a secondary anchor in the wrong location by the correct gRNA could lead to the formation of an unexpected editing pattern, which would terminate the editing process since a correct anchor for the next gRNA would not be formed. However, misedited sequences within the junction region could be re-edited with the correct gRNA. Many examples of misediting/misguiding

which are consistent with this hypothesis have been found. However, another interpretation of unexpected patterns was proposed by Decker and Sollner-Webb (Decker and Sollner-Webb 1990b). They suggested that editing is completely random and occurs between every nucleotide within an editing domain, and that when the correct sequence is formed it is "frozen" by the formation of base pairs with the gRNA. This issue will not be resolved until we have a complete knowledge of the total gRNA content of the mitochondrion and can compare all unexpected patterns to known gRNA sequences, and also a deeper understanding of the mechanism of editing.

The enzyme cascade model is consistent with most observations, including the known 3′ to 5′ polarity of editing, but it does not really explain the existence of the 3′ oligo-[U] tail on the gRNA (Blum and Simpson 1990). We had proposed a role for the 3′ oligo-[U] tail in stabilizing the initial hybrid, since the U's would form base pairs with G's and A's in the pre-edited region. However, soon after we had proposed the "enzyme cascade" model, Blum, who had this problem of always thinking too much, had the idea that perhaps the oligo-[U] tail played a more active role and actually was the source of the U's added during editing. We therefore proposed another model in which the 3′ terminal OH of the gRNA attacked a phosphate within the mRNA at the site of the first mismatch between the gRNA and mRNA, resulting in exchange of the OH for the phosphate by transesterification (Blum and Simpson 1992; Blum et al. 1991). The transesterification reaction is similar to that which occurs in self-splicing of RNA molecules in other cells. A prediction of this model is the existence of intermediate chimeric molecules which consist of gRNAs covalently linked to mRNAs at editing sites by the 3′ oligo-[U] tail. Blum, Sturm and Agda Simpson immediately searched for and found these chimeric molecules for three genes. This was gratifying, but did not really prove the transesterification model, since chimeric molecules could possibly be formed in other ways, especially in a system which we knew contains a cleavage activity and an RNA ligase activity.

At about this time I visited Tom Cech in Boulder. Cech, who had discovered that RNA could have enzymatic activity, told me that he had solved the editing problem and proceeded to draw on a sheet of paper the entire transesterification model! (Cech 1991). As happens frequently in science, when ideas are ripe, they germinate simultaneously in several gardens. The transesterification model was theoretically attractive since it employed the same chemistry and the same type of guide sequences used in the well understood self splicing of introns, whereas the enzyme cascade model was a novel set of protein-mediated reactions. However, this was an example of a beautiful theory which crashed on the hard rocks of facts, since it turned out not to be true (Frech and Simpson 1996). Our original cleavage-ligation model has proved to be essentially correct in almost all details.

Evolution of RNA Editing in Trypanosomes

The question whether RNA editing is a primitive or derived mechanism is an important one and yet to be resolved. To address this question we looked at *Trypanoplasma borreli,* the only species in the group of bodonids which could be grown in axenic culture. This paraphyletic group is related to trypanosomatids and is thought to have evolved prior to the emergence of the monophyletic trypanosomatids. *T. borreli* turned out to have two classes of molecules in the mitochondrion: 80 kb circles which encode homologues of trypanosome maxicircle genes, and 260 kb megacircles which encode guide RNA genes (Fig. 11) (Maslov and Simpson 1994). Other labs showed that several other bodonid species also contain maxicircle homologues and non-catenated minicircles with gRNA genes. It appears that the separation of the mitochondrial genome into maxicircle-encoded cryptogenes and minicircle- or megacircle-encoded gRNA genes occurred in the bodonid lineage, and catenation of minicircles to form the kDNA network first evolved in the ancestor of the trypanosomatids (Fig. 12). A postdoctoral fellow from Japan, Shinji Yasuhira, and Maslov also examined mitochondria of *Euglena* and *Diplonema*, the other member of Euglenozoa (Maslov et al. 1999; Yasuhira and Simpson 1997). No evidence for editing was found but it is hard to prove a negative. But since this type of RNA editing is not seen in other eukaryotes, it is likely that U-insertion/deletion editing arose in the mitochondrion of the ancestor of the kinetoplastid protists.

Maslov and I also did a comparative study of editing of several genes in different trypanosomatid species (Simpson and Maslov 1994). We found a gradual loss of editing from the 3' end and we speculated that this was due to the loss of specific gRNA-encoding minicircle classes in evolution, which could be compensated by retroposition of cDNAs of partially edited mRNAs replacing the original pan-edited cryptogenes in the maxicircle

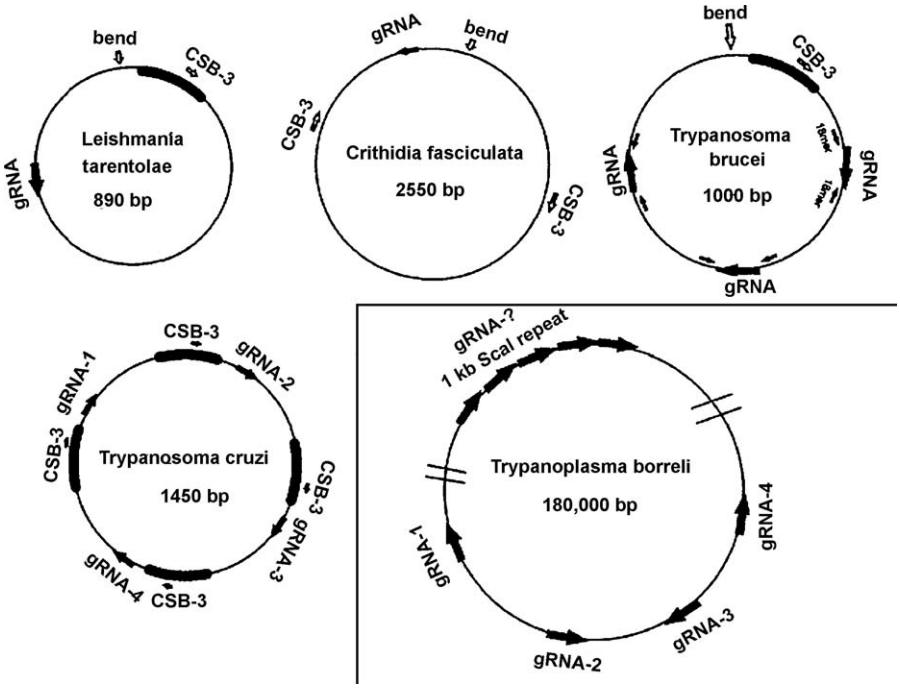

Figure 11. Comparison of gRNA gene organization in several trypanosomatid species and in the bodonid, *Trypanoplasma borreli*. From Mol Biochem Parasitol (1997) **86**: 133-141, with permission.

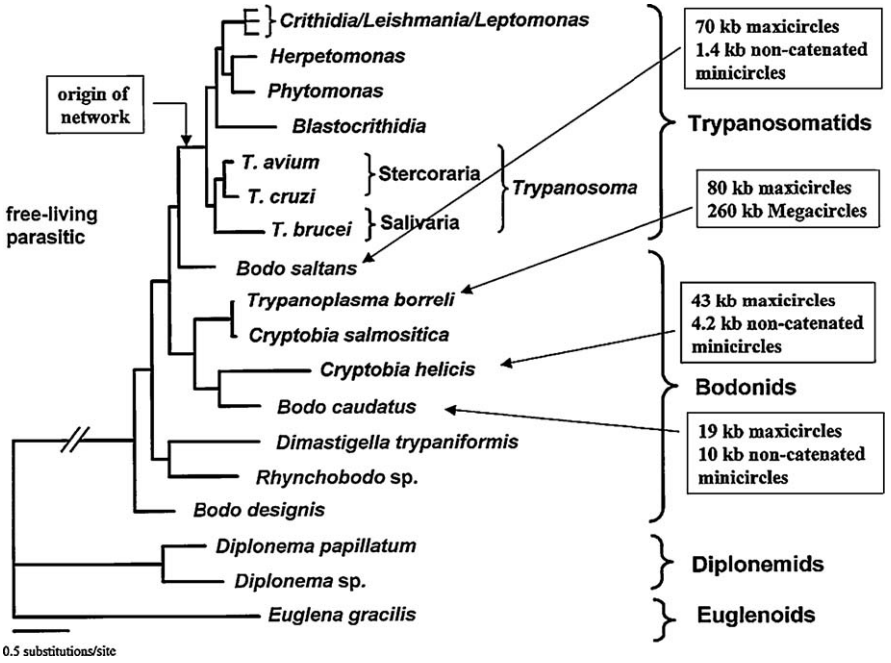

Figure 12. Phylogenetic tree of Kinetoplastida based on SSU rRNA sequences. Maximum likelihood method. Some unpublished data of D. Dolezel, M. Jirku and J. Lukes, with thanks. From Proc Natl Acad Sci USA (2000) **97**: 6986-6993, with permission.

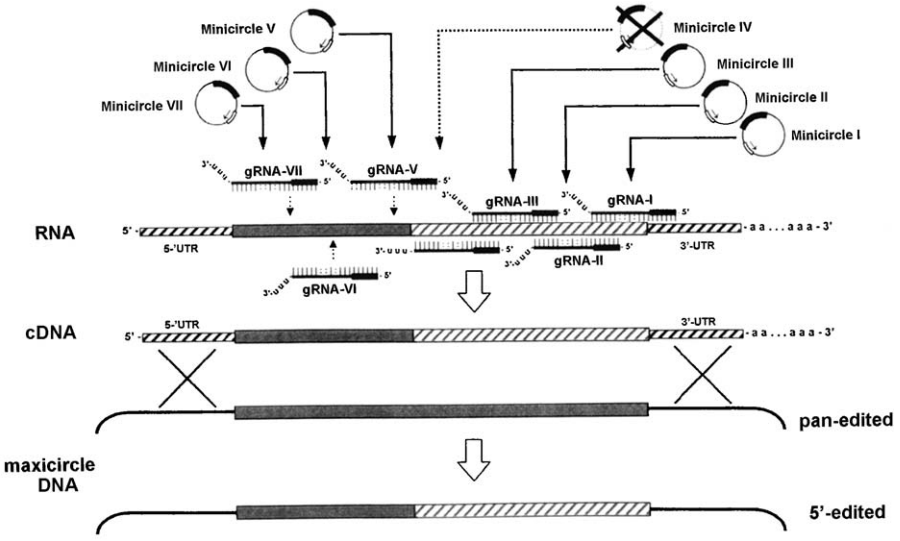

Figure 13. Retroposition model for evolution of RNA editing. A pan-edited domain mediated by 7 overlapping gRNAs is shown. Loss of minicircle class IV indicated by crossing out. Proposed double crossover of partially edited cDNA and pan-edited maxicircle gene indicated by X. From Annals NY Acad Sci (1999) **870**: 190-205, with permission.

genome (Fig. 13). This model fits all available data, but raises the unsettling question of why editing has been maintained at all in evolution.

All tRNAs are Imported into the Kinetoplast-Mitochondrion of Trypanosomes

During the decade from 1990 to 2000, we were occupied trying to develop in vitro editing reactions and to learn more details of the reaction mechanism. In addition we started a side project which was based on our discovery that there were no tRNA genes in the maxicircle. A Brazilian postdoctorate, Beatriz Lima, showed that *L. tarentolae* cells could be transfected with a plasmid expressing mitochondrial tRNAs and these would enter the mitochondrion in vivo (Lima and Simpson 1996). A graduate student, Mary Anne Rubio, subsequently developed an in vitro import system (Rubio et al. 2000) using isolated kinetoplast-mitochondria and showed that different tRNAs had different specificities. Another graduate student, Steve Kapushoc, did a quantitative assay of the localization of tRNAs in the cytosol or mitochondrion (Kapushoc et al. 2002).

The mitochondrial genetic code in trypanosomes as in other organisms uses the UGA stop codon as tryptophan, and the importation of all tRNAs into the mitochondrion presented a theoretical problem for decoding UGA. Juan Alphonzo, then a postdoctorate in my lab and now a Professor at Ohio State University, had the ingenious realization that a possible solution would be that the imported $tRNA^{Tryp}$ undergoes a single C to U substitution or editing event in the first position of the anticodon (Fig. 14) (Alfonzo et al. 1999). This, surprisingly, turned out to be true and was the first indication that C to U editing occurs in trypanosomes as in several other organisms.

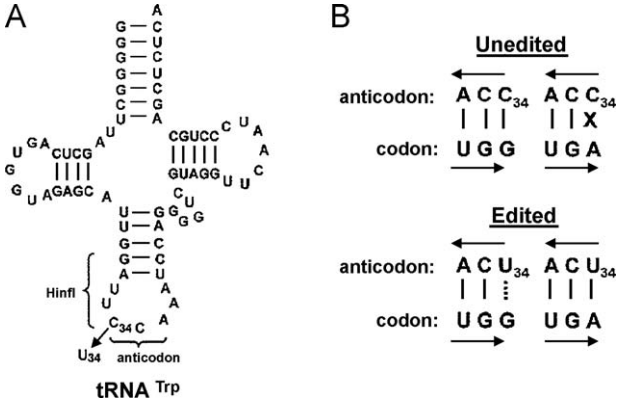

Figure 14. C to U editing of the anticodon of the mitochondrial-imported $tRNA^{Trp}$. **A.** Editing of C34 leads to destruction of HinfI site. **B**. C34 to U34 editing allows decoding of UGA codon as tryptophan. From EMBO J (1999) **18** 7056-7062, with permission.

Biochemistry of Editing Enzymes

The editing project received a boost when Ruslan Aphasizhev, then a postdoctorate and now a Professor at the University of California at Irvine, and Marian Peris, a graduate student, initiated a major lab project to biochemically isolate the 3' TUTase from *L. tarentolae* mitochondria. This project involved the development of large scale culture and mitochondrial isolation methods and was ultimately successful (Aphasizhev et al. 2002). Interestingly, this TUTase, which we have termed RET1, turned out to be responsible for the 3' addition of U's to gRNAs (Aphasizhev et al. 2003c).

Then we decided to utilize a new double epitope tagging technique, the TAP method, to isolate the editing complex. Several labs had at that time cloned and sequenced the two mitochondrial RNA ligases, REL1 and REL2, and, based on this sequence data, we transfected *L. tarentolae* cells with a REL1-TAP fusion protein construct. This led to the isolation of a high molecular weight complex (Aphasizhev et al. 2003a) containing at least 16 proteins which we called the L-complex and now call the RNA Editing Core Complex or RECC (Simpson et al. 2009). Utilizing the power of mass spectrometry combined with the *Leishmania major* genome project, we were able to identify all of component proteins. One of the proteins was identified as a 3' TUTase, and this enzyme, which we termed RET2, proved to be responsible for the insertion of U's at editing sites (Aphasizhev et al. 2003c). At the same time we isolated the MRP RNA-binding complex, and showed that this and also the RET1 complex interact via RNA linkers with the RECC (Aphasizhev et al. 2003b). All these results were and are very exciting and we and others have worked extensively on the molecular biochemistry of the editing machinery.

My current research project is to determine the molecular structure of the *L. tarentolae* RECC (Li et al. 2009). We are doing this in a close collaboration with the lab of Hong Zhou at UCLA, a fantastic cryo-electron microscopist with some equally fantastic microscopes.

Epilogue

A scientific career is somewhat like a river that starts as a small creek, grows as more creeks join it, occasionally meanders and changes direction, and often splits into separate streams, some of which continue downhill and others end in the fens. My career has been somewhat unusual in that it has been monomaniacally focused throughout on a single biological phenomenon, the kinetoplast-mitochondrion of the kinetoplastid protists. But my interests have changed from basic parasitology and cell biology, to DNA structure and replication, to transcription and RNA biology, and finally to protein biochemistry. It is hard to summarize a life in science that is still actively progressing, but I can say that it has been a lot of fun and that I was very lucky to have such excellent graduate students and postdoctoral fellows to share the excitement of the adventure.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2010.08.001.

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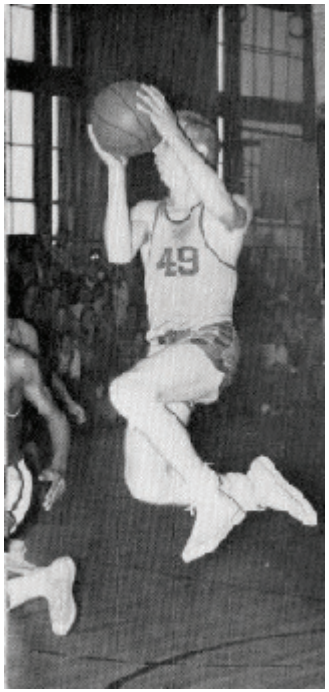


Supplemental Figure S1

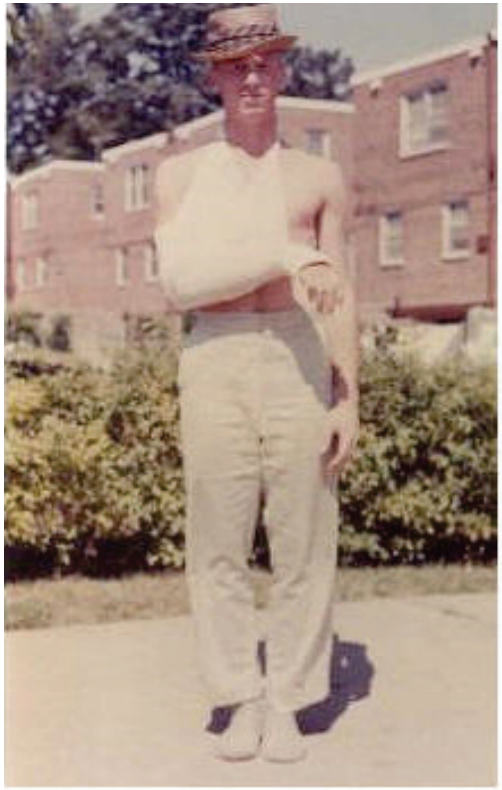
Supplemental Figure S2

LARRY P. SIMPSON

It has often been said that if there are two ways to do it, Larry will do it his way ("Where art for thou Romeo" e.g.). Simpson and inverted word order are synonymous. But Larry has been noticeable in other fields. His action on the basketball floor and in competitive examinations has made him a center of attraction.

Barnwell and Scholastic Honors; Cadet Basketball, JV Basketball, Varsity Basketball; Intramural Sports; College Entrance (Chairman), Prom, Social, Record Book, *Tutoring Committees; Math Club,* Mirror, *Camera Club; Student Tutor; SA Representative.*

Supplemental Figure S3



Supplemental Figure S4

Supplemental Figure S5

Supplementary Figure S6

Supplementary Figure S7