EVOLUTION and the MOLECULAR REVOLUTION

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CHAPTER 6

FROM MOLECULAR EVOLUTION TO BIOMEDICAL RESEARCH: THE CASE OF CHARLES DARWIN AND CHAGAS' DISEASE

Larry Simpson*

DARWIN'S ILLNESS

Charles Darwin (Figure 6.1) has always presented a fascination for everyone interested in the history of science, especially those interested in development of the theory of evolution. Here was a man who single-handedly changed the conceptual framework of all biological science and the way we look at the living world around us, but who led a quiet, outwardly uneventful life. In fact, the one aspect of Darwin's personal life that was unusual was his state of health. For over half his life, Darwin suffered from an undiagnosed chronic illness that severely affected both his ability to do science and his interactions with people in general. His symptoms, which included extreme fatigue and digestive and intestinal problems, were frequently almost incapacitating. That Darwin was able to produce such an impressive body of work is truly amazing.

For the first 27 years of his life, until 1838, Darwin was in excellent health. In the summer of 1826, Darwin and two friends took a walking tour through North Wales, often covering 30 miles a day and climbing local peaks (Adler, 1989). At Cambridge University, Darwin was a member of the Gourmet Club and his diary includes many entries about eating and drinking (Adler, 1989). As the ship's naturalist on the *H.M.S. Beagle* during a voyage that lasted from 1831 to 1836 (Figure 6.2), Darwin was considered one of the most fit members of the crew and he frequently went ashore and undertook long overland expeditions (Adler, 1989). His only illness during this trip (aside from continual sea sickness) was a month-long bout of fever of unknown origin in 1834.

In 1836, Darwin returned to England and was in good health for the next two years until his chronic symptoms began to appear and his life changed permanently. In 1838, he refused the secretaryship of the Geological Society of London for reasons of ill health,

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FIGURE 6.1

Charles Darwin in 1840 at age 31, shown in a watercolor portrait by George Richmond. (Reproduced, with permission, from Moorehead, 1969.)

and was advised by his doctors to stop working temporarily (Goldstein, 1989). Darwin's health became progressively worse during the next few years. He suffered from periodic vomiting and debilitating fatigue. In 1839, he married, and in 1842, his family moved to a country home, thinking that the peaceful surroundings would improve his health. However, he wrote to botanist Joseph Hooker in 1845:

> I believe that I have not had not a whole day, or rather night, without my stomach being greatly disordered, during these last three years, and most days great prostration of strength.

Darwin also recorded the fact that after social dinners with his friends he commonly suffered "violent shivering and vomiting attacks" and that because of this unpleasantness he was "compelled for many years to give up all dinner parties." In 1849, he was too ill to attend his father's funeral. He wrote: "I was almost quite broken down, head swimmy, hands trembling and never a week without violent vomiting."

Throughout his life, Darwin was examined by many eminent physicians who could find no organic cause of his symptoms. During his last ten years, from 1862–1872, Darwin's health improved somewhat, but his symptoms never fully disappeared.

Many theories have been proposed to account for Darwin's mysterious illness. These range from suggestions that he was a hypochondriac, that he suffered an Oedipal complex or a psychosis, that he had an allergy to pigeons—even that he was being poisoned by the arsenic in his medications—to the idea that he had an undiagnosed chronic disease such as brucellosis, a bacterial infection (Goldstein, 1989).

CHAGAS' DISEASE

In a compelling paper published in 1959, Saul Adler, an Israeli parasitologist, suggested that Darwin suffered from chronic Chagas' disease and that he contracted this disease during the voyage of the *Beagle* (Adler, 1959). Chagas' disease is an incurable chronic disease caused by the **eukaryotic** (cell nucleus-containing) protozoal parasite, *Trypanosoma cruzi*. It affects the heart, the involuntary **(autonomic)** nervous system, or both, and is a major cause of sudden death in Latin America. Approximately 16 to 18 million people inhabiting the region from Argentina to Mexico are infected with this parasite.

The *T. cruzi* parasite belongs to a large group of flagellated protozoa known as **kinetoplastid** protozoa, named for the presence of a large mass of circularly organized DNA (kinetoplast DNA) that occurs in the energy-producing organelle (**mitochondrion**) at the base of the whiplike **flagellum** that enables these protozoans to swim (Simpson, 1987; Simpson, 1972) (Figures 6.3 and 6.4). This DNA consists of a giant network of thousands of interlocked small circles, **catenated minicircles**, and a smaller number of larger catenated **maxicircles** (Figures 6.5 and 6.6). This may be one of the most unusual DNA structures known in nature; it has been the subject of intensive research in several laboratories, including my own.

Chagas' disease was discovered in 1909 by the Brazilian naturalist, Carlos Chagas, who found the parasites in insects and other animals and decided to look for them in sick humans (Figure 6.7). Chagas completely described both the life cycle of the parasite in the **insect vector** (the host organism of the parasite that transmits the parasite to vertebrates at one stage in its life cycle) and in the human or animal host. He also described in detail the epidemiology of the disease and the pathology it caused (see Prata, 1981, for a collection of Chagas' papers). Chagas' disease is one of only a few examples of a human disease that was discovered *after* the disease-causing parasite had been found in nature. Chagas named the parasitic species *T. cruzi*, in honor of Oswaldo Cruz, another distinguished Brazilian scientist. Chagas' work led to the establishment of a Brazilian tradition

FIGURE 6.2

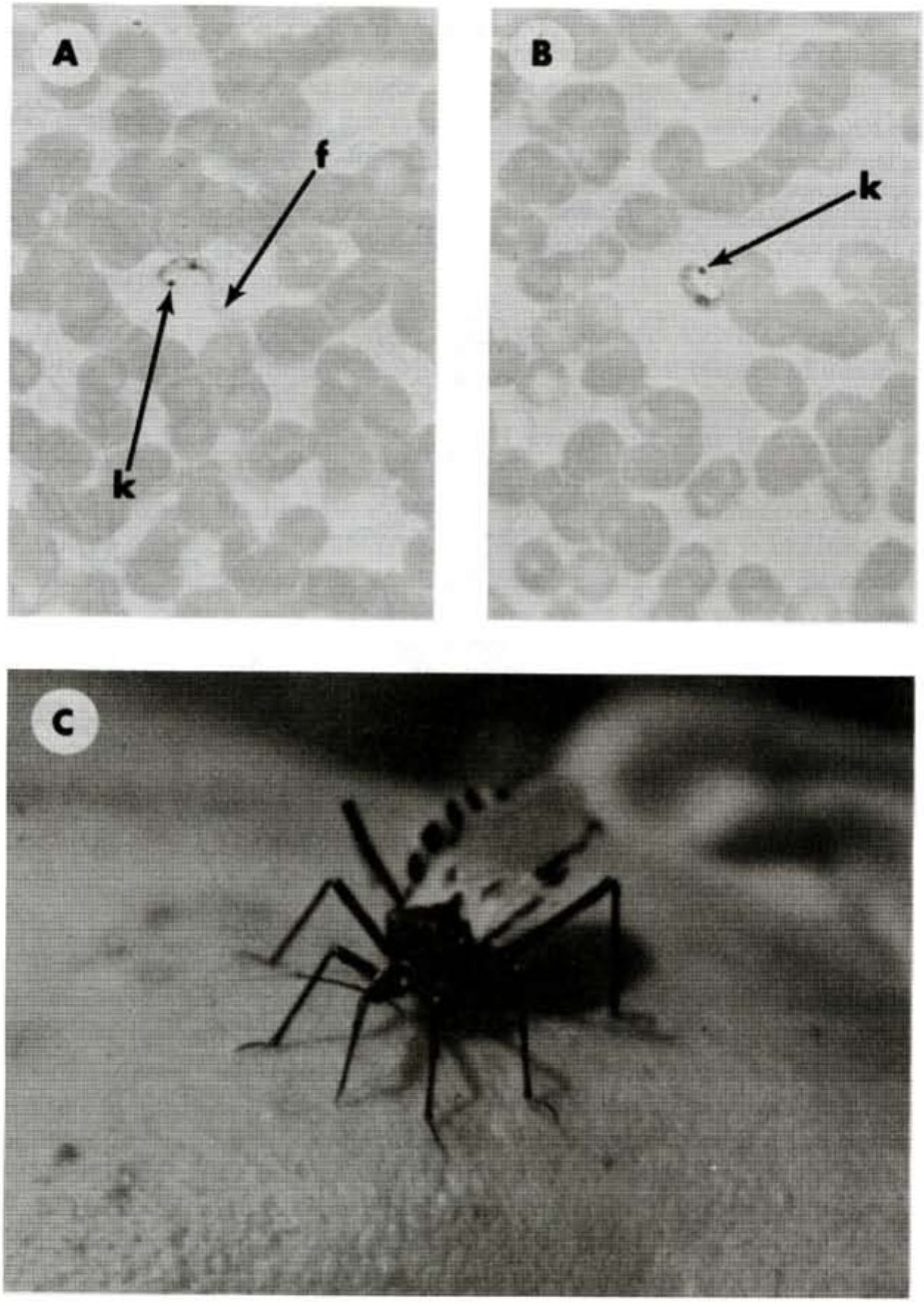
Map of a portion of the Beagle's voyage, showing Darwin's land excursions in South America. (Reproduced, with permission, from Stone, 1980.)

of research into the biology, biochemistry, and, recently, the molecular biology, of these widespread parasites.

The parasite is transmitted by **reduviid bugs**, a particular type of insect belonging to the genera *Triatoma, Rhodnius,* and *Panstrongylus*. These insects live in the walls and thatched roofs of adobe huts, and they emerge at night to feed on sleeping people (Figure 6.3C). In Brazil, the insects are known as "kissing bugs," because they frequently bite the face of their victim near the mouth or eyes, or as "barber bugs," because they engorge themselves with blood like the leeches used by ancient barbers. Their bloodsucking has also caused them to be labeled "assassin bugs." The *T. cruzi* parasites living in the reduviids are excreted in bugs' feces, which they deposit soon after a blood meal; a sleepy person who has been bitten may rub the parasite-containing fecal material into the wound and thereby initiate the infection.

Some term Chagas' disease a *social disease*, because better living conditions eliminate the insect vector and stop the normal mode of transmission. However, even with improved living conditions, transmission by blood transfusions or from a mother to her unborn fe-

FIGURE 6.3



Trypanosoma cruzi and its insect vector. Parts A and B: Light photomicrographs showing *T. cruzi* in a stained blood film from an infected person; note the intensely stained kinetoplast (k) at the base of the flagellum (f). Part C: A reduviid bug taking a blood meal; reduviids in the genera *Triatoma*, *Rhodnius*, and *Panstrongylus* transmit *T. cruzi* by fecal contamination. (Reproduced, with permission, from Peters and Gilles, 1977.)

tus would still be major problems. In fact, transmission by transfusion has recently become a problem in the Los Angeles Blood Bank because of donation of blood by infected people who have immigrated from Central America (Kirchoff, 1989; Kirchoff et al., 1987).

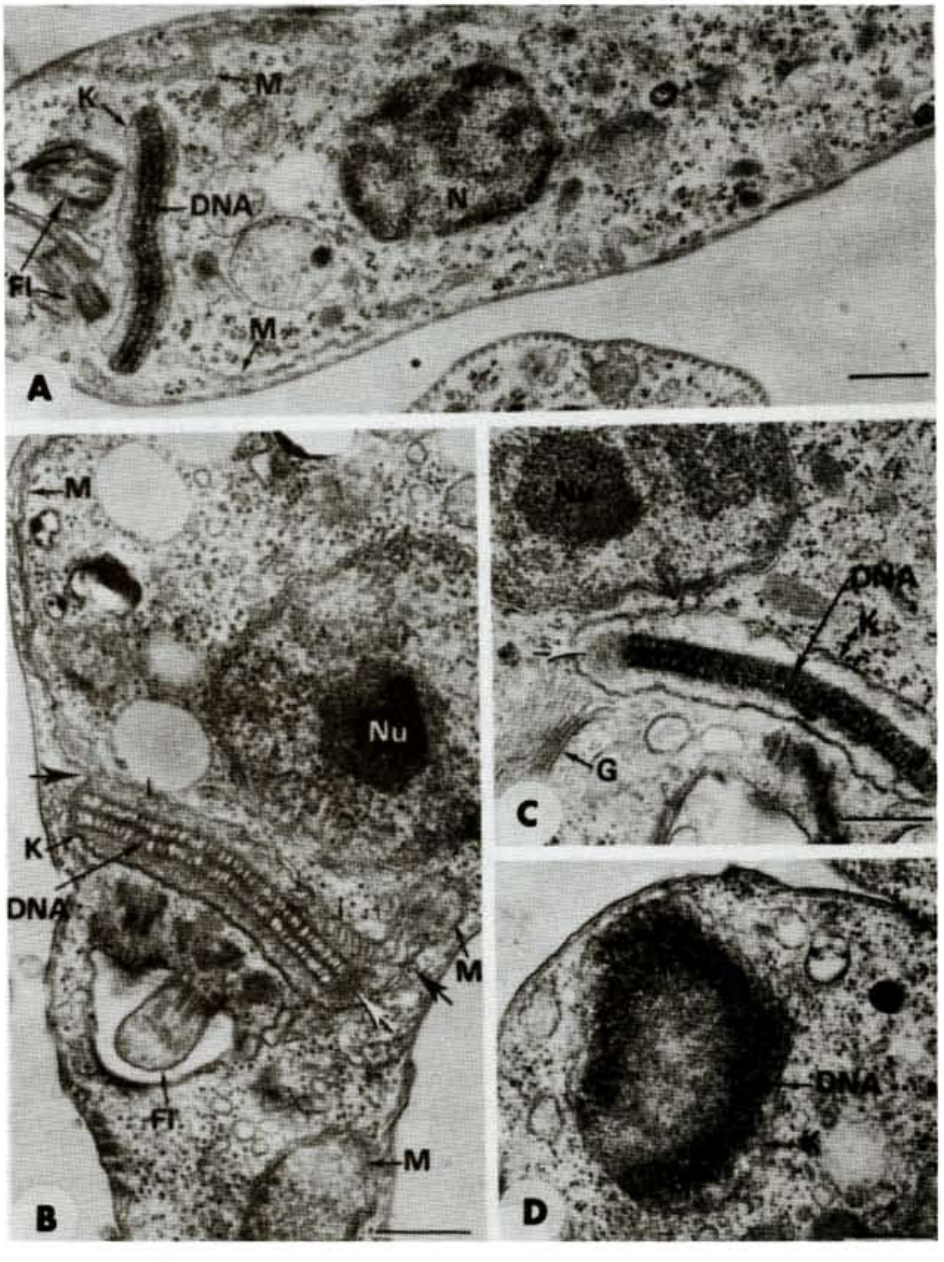
Once inside the host, the *T. cruzi* parasites migrate rapidly through the blood to cells of heart muscles or of the autonomic nervous system, which they penetrate and in which they survive and live as intracellular parasites. The acute phase of Chagas' disease usually occurs in children and involves fever, swelling of the lymph nodes, spleen, and liver, and serious damage to the heart (myocarditis). Some mortality occurs in the acute phase, but most people survive and the symptoms disappear after several months. Approximately 20% to 40% of infected people develop the chronic form of the disease (Schmunis, 1991), which involves progressive degeneration of the heart, of the gastrointestinal system, or of both. After infection, there is usually a lag period of three to five years before the onset of the chronic symptoms, which last for the rest of the life of the diseased victim.

The "Benchuca" Incident on the Voyage of the *Beagle*

Adler (1959) noted that many of the symptoms Darwin reported matched those of chronic Chagas' disease. Moreover, Adler found written on March 26, 1835, in Darwin's chronicle of the expedition, *The Voyage of the Beagle*, a passage describing an overland trip Darwin took from Chile across the Andes Mountains to the plains of Argentina (Figures 6.2 and 6.8):

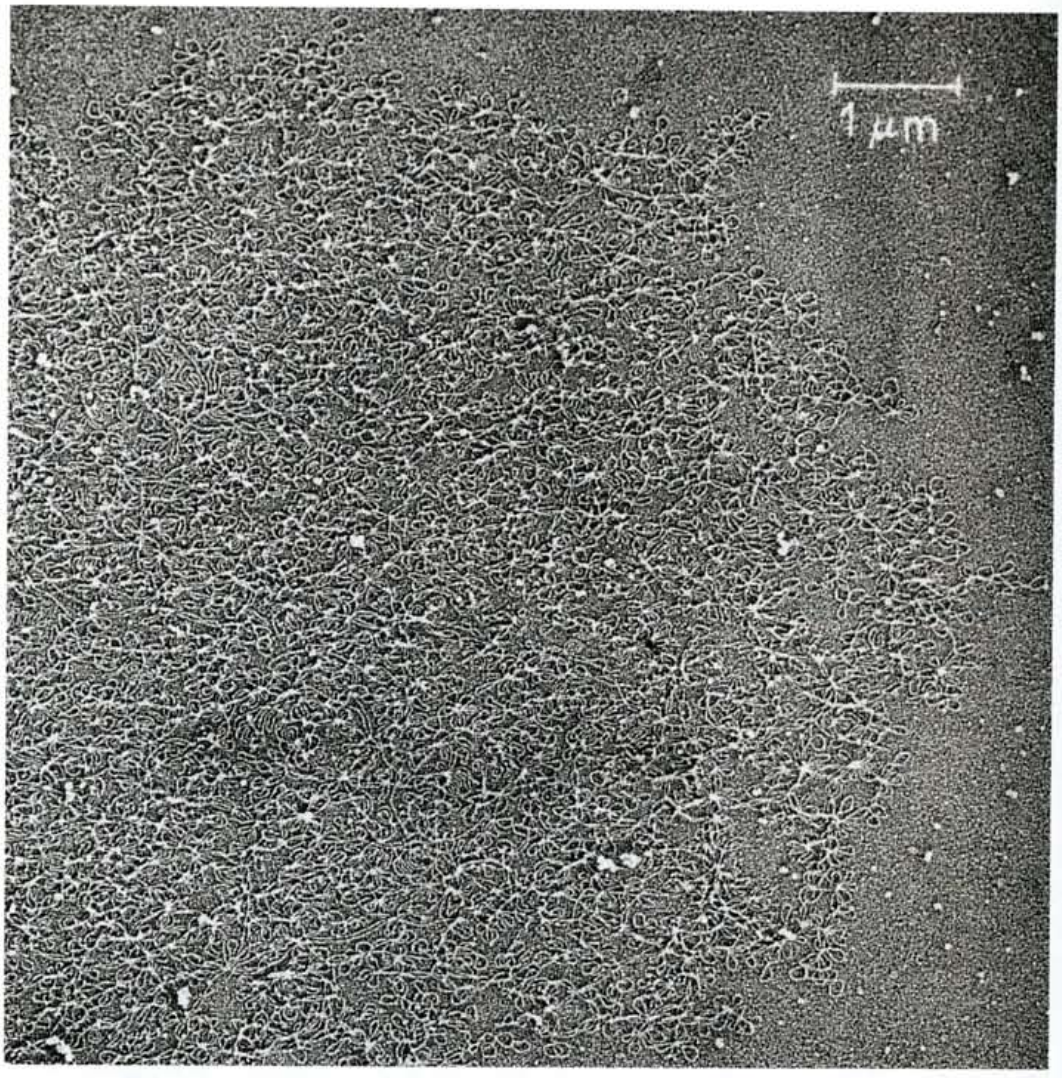
> We slept in the village, which is a small place, surrounded by gardens, and forms the most southern part, that is cultivated, of the province of Mendoza; it is five leagues south of the capital. At night I experienced an attack (for it deserves no less a name) of the Benchuca (a species of *Reduvius*) the great black bug of the Pampas. It is most disgusting to feel soft, wingless insects, about an inch long, crawling over one's body. Before sucking, they are quite thin, but afterwards be-

FIGURE 6.4



Electron micrographs of kinetoplast DNA in *Trypanosoma cruzi* cells. Parts A to C: Longitudinal sections through the kinetoplast-containing (K) portion of the single mitochondrion, showing the kDNA (DNA), mitochondrial tubules (M), nucleus (N), flagellum (Fl), nucleolus (Nu), and Golgi (G); the white arrow in the lower right portion of Part B points to an amorphous mass of kDNA at one end of the nucleoid body, which may represent a site for minicircle DNA replication. Part D: Transverse section through the kinetoplast nucleoid body showing the minicircular DNA fibrils. (Reproduced, with permission, from Delain and Riou, 1969.)

FIGURE 6.5



Electron micrograph of a kinetoplast DNA network from *Leishmania tarentolae*. The DNA was dispersed on water, picked up on a carbon surface, and shadowed with platinum/paladium. The interlocked (catenated) minicircle and maxicircle DNA molecules are well defined (but are especially evident at higher magnification, as shown in Figure 6.6). (Reproduced, with permission, from Simpson and Berliner, 1974.)

come round and bloated with blood, and in this state they are easily crushed. They are also found in the northern parts of Chile and in Peru.

The "great black bug of the Pampas" was probably the reduviid *Triatoma infestans*—more than 70% of specimens of that species in that region of Argentina are infected with the *T. cruzi* parasite at the present time (Schmunis, 1991). In addition, approximately 12% of the human population today living in that region have been shown to contain **antibodies** (biochemicals of the immune system) against *T. cruzi* (Schmunis, 1991). This passage provides one specific instance in which Darwin could have been infected with *T. cruzi*. Furthermore, the three- to five-year period until the onset of his symptoms agrees with the known lag period for the onset of chronic Chagas' disease.

Diagnosis of Chagas' Disease

Adler's theory has created much controversy and has not been accepted by everyone, mainly because it has been impossible to verify (Adler, 1989; Bernstein, 1984; Goldstein, 1989). Chagas' disease was undescribed until the early 1900s, more than 70 years after Darwin visited the Argentinian Pampas, and even today it is difficult to diagnose in chronic patients because the parasites are normally localized within cells of heart and nerve tissue; only very few parasites circulate in the blood. Remarkably, the best method for clin-

ical diagnosis of the presence of these parasites in a patient is **xenodiagnosis**, in which uninfected kissing bugs are actually allowed to feed on a patient (!), are grown in the laboratory for several weeks, and are then dissected to see whether parasites are present in their hind guts (Figure 6.9). Although this method (aside from having obvious esthetic problems) lacks sensitivity and reproducibility, it nevertheless is the current gold standard for detection of parasites in a patient. Detection of antibodies against the parasite is also used, but the reagents employed frequently react with other antibodies as well, and therefore the test is not entirely reliable.

KINETOPLAST DNA: MINICIRCLES AND MAXICIRCLES

This laboratory has been involved in studies of the genetic material (the "genome") of the kinetoplast of the mitochondrion (see Box 6.1) in kinetoplastid protozoa since 1969. In 1980, a collaboration began with Carlos Morel from the Oswaldo Cruz Institute in Rio de Janeiro on the kinetoplast DNA (**kDNA**) of *T. cruzi*. From 1917 to 1934 Carlos Chagas, the discoverer of Chagas' disease, directed the Oswaldo Cruz Institute (Figure 6.10), and Morel is currently President of the Oswaldo Cruz Foundation that administers the In-

FIGURE 6.6

Electron micrograph of a fragment of a kinetoplast DNA network from *Leishmania tarentolae*, showing catenated minicircles and "figure of eight" DNA molecules (which may be minicircle recombinants). The precise molecular topology within the network is not understood. (Previously unpublished electron micrograph provided by Dr. Agda Simpson, University of California, Los Angeles.)

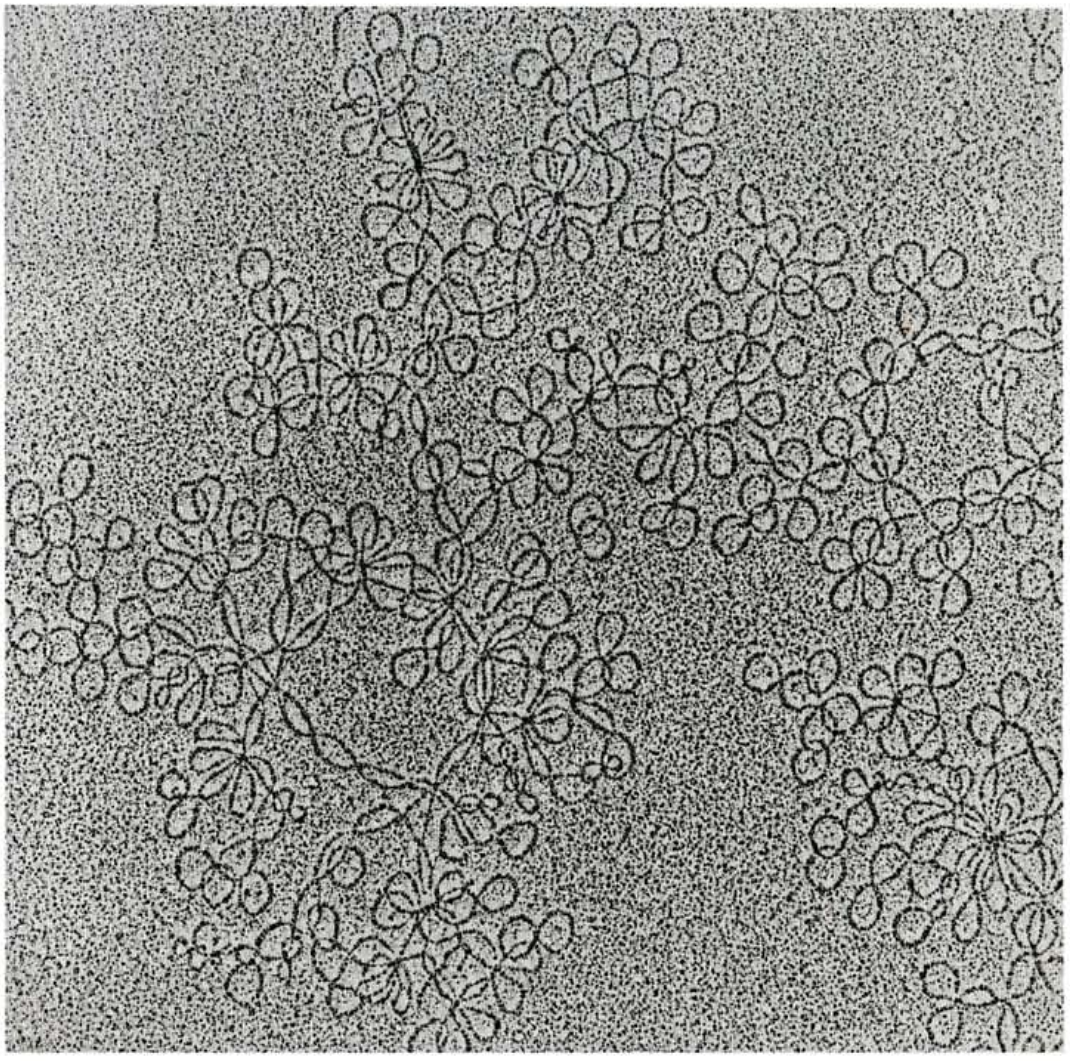


FIGURE 6.7

Carlos Chagas (center) and members of an expedition from the Oswaldo Cruz Foundation on the Rio Negro River in Amazonas, Brazil, 1913. (Reproduced, with permission, from Thielen, 1991.)

stitute. Morel and I found that the kDNA from different strains of *T. cruzi* differed greatly in the sequence of the subunits of which they are composed (Morel et al., 1980). This was shown by digesting the kDNA with special enzymes (**restriction enzymes**) that cleave DNA at specific places in the molecule and, as illustrated in Figure 6.11, by separating the cleaved fragments with use of high resolution **gel electrophoresis** (a technique in which the cleaved segments are subjected to an electrical charge that pulls them across a gel; because short pieces move faster than longer ones, the fragments are separated into a pattern of bands that will be identical from one sample to another only if the kDNAs are the same). Because the minicircle DNA makes up 95% of the total kDNA network, we believed that the sequence differences detected were caused by the DNA in the minicircles. We coined the term, **schizodeme**, to refer to various strains that distinctly differ one from another in their kDNA minicircle sequences (Morel et al., 1980). The genetic reason for the large amount of sequence divergence was shown only recently in genetic studies by Tibayrenc, Ayala, and colleagues to be a result of the fact that the various *T. cruzi* schizodemes actually represent major evolutionary lineages, genetically isolated from each other for such long periods of time that the minicircle sequences have diverged extensively (Tibayrenc et al., 1986; Tibayrenc et al., 1990).

In the early 1980s, we did not know the genetic function of the minicircle molecule, which is present in high abundance in the kinetoplast. The other molecular species in the network, known as maxicircle DNA, appeared to represent the evolutionarily related **ho-**

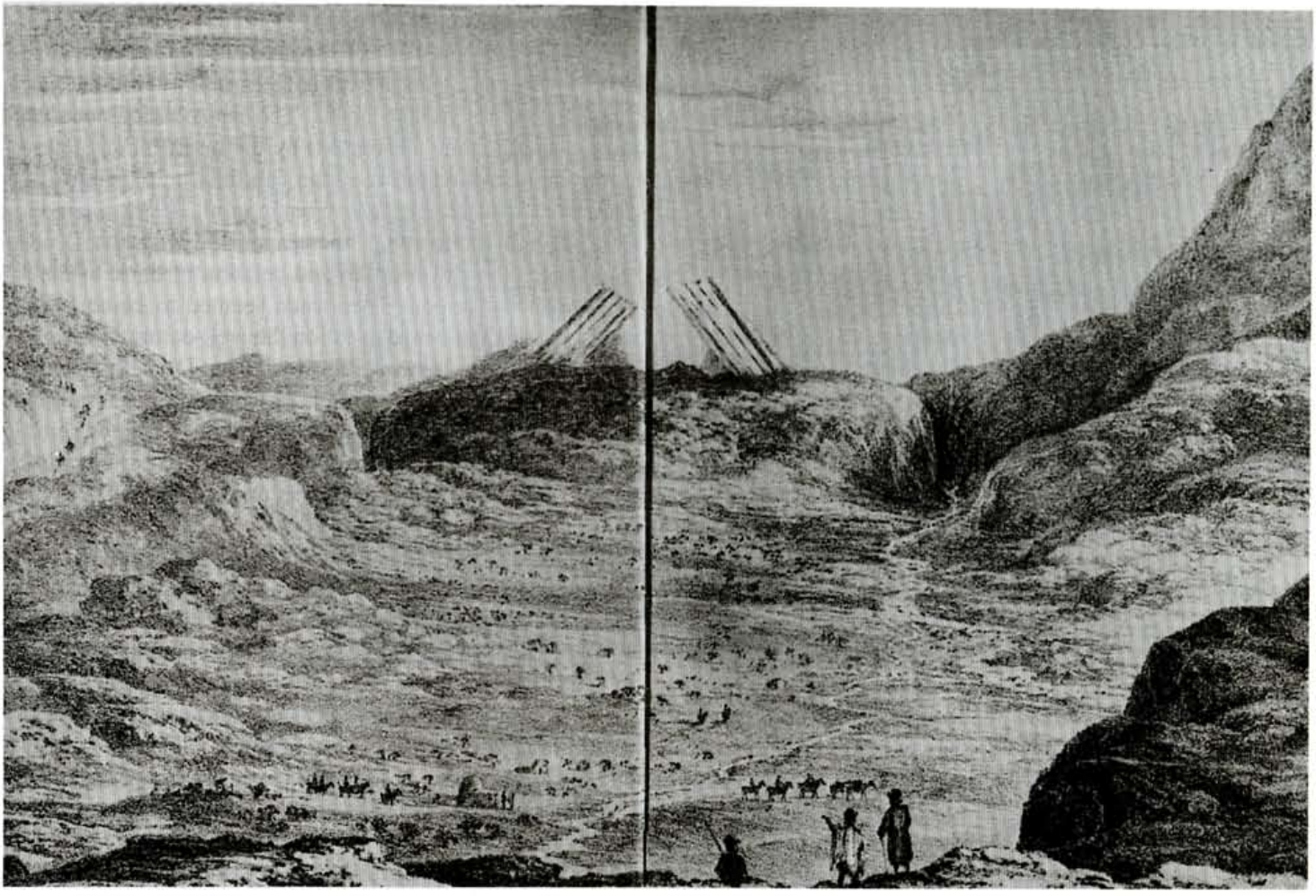


FIGURE 6.8

A long, narrow basin separating the Uspallata Range from the Andes mountain chain, north of Mendoza, Argentina, near where Darwin stayed overnight and was bitten by a "Benchuca" bug. The curious structure in the distance probably represents an uplifted rock unit. (Reproduced, with permission, from Moorehead, 1969, after Schmidtmeyer, 1824.)

mologue of the informational DNA molecule found in human mitochondria (Simpson, 1987).

RNA EDITING

The DNA strands that contain genetic information in cells are each made up of nearly three billion subunits, called **nucleotides**, each of them representing one of the four "letters" in an information-containing chemical code (A, adenine; T, thymine; C, cytosine; and G, guanine). One of the basic tenets of molecular genetics is that the nucleotide sequence in the DNA should be copied faithfully, as determined by rules of chemical bonding (the ability to make base pairs, discussed in Chapter 2), into a molecule known as **messenger RNA (mRNA)**—the molecule that carries the coded message from the DNA to ribosomes in which proteins are manufactured. The mRNA copies should be perfect, except for lacking intervening sequences (stretches equivalent to the "junk" DNA that is

FIGURE 6.9

Xenodiagnosis. Uninfected reduviid bugs are permitted to take a blood meal on the patient, and are then grown in the laboratory for several weeks and examined for the presence of *Trypanosoma cruzi* in their hind guts. (Reproduced, with permission, from Peters and Gilles, 1977.)

interspersed between information-containing segments in the genome) and having an added 3′ **poly[A] tail** and an added 5′ **cap structure** at their ends. In the late 1980s it was discovered that, in addition to normal genes, the maxicircle DNA molecule in the mitochondrion of kinetoplastid protozoa contains hidden genes. The RNA strands copied from these **cryptogenes** are modified (that is, they are **edited**) by the insertion and deletion of **uridine** (U) residues, and the modified, edited message can then be translated at ribosomes to produce protein products (Benne et al., 1986; Feagin et al., 1988; Shaw et al., 1988; Simpson et al., 1993; Stuart, 1993). However, there appeared to be no DNA source for the new information represented by the addition and deletion of these uridine residues. The information for the modified nucleotide sequence seemed to come from nowhere! These surprising results led some scientists to question the well-entrenched **central dogma** of molecular genetics—that genetic information flows from DNA to mRNA and from mRNA to protein—because the genetic information for the sequence changes in the mRNAs (information that determines the sequence of amino acids in the proteins they produce, discussed in Chapter 2) did not appear to reside in the DNA of the organism. We now believe that these results can be explained in terms of a model that is consistent with the central dogma.

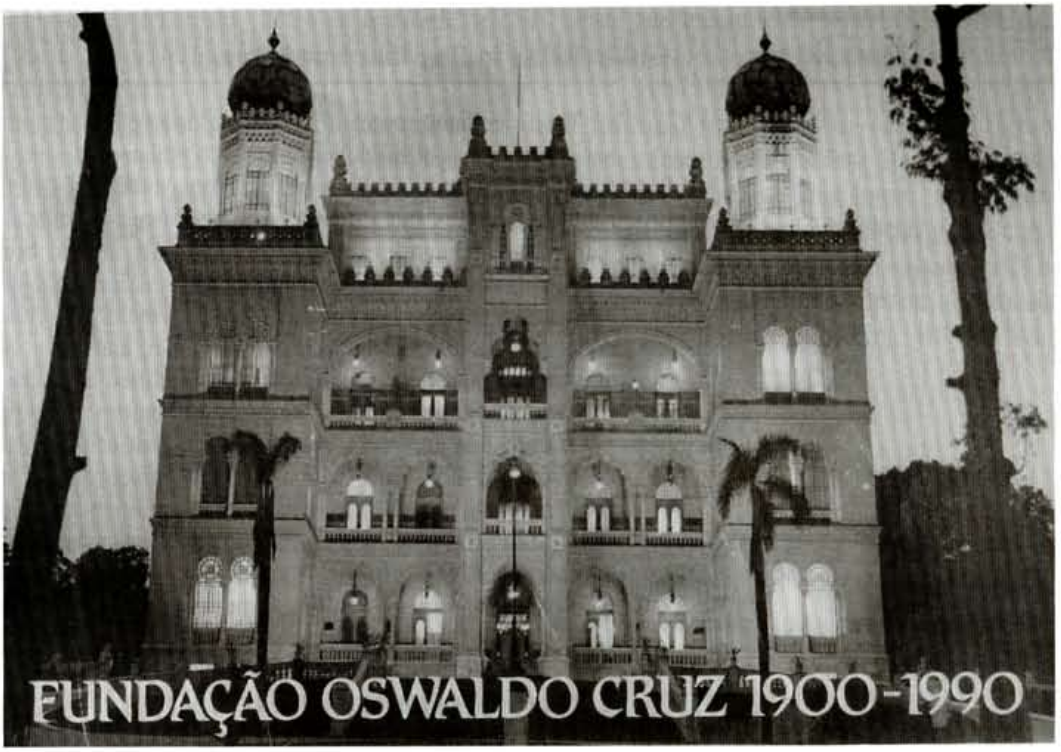
Central to this model is the existence of a new class of RNA molecules, known as **guide RNAs (gRNAs)** (discussed in Box 6.2), that contain short pieces made up of the information required to produce the edited nucleotide sequences. We discovered gRNAs in 1989 by performing a computer search of the known maxicircle sequence, looking for short stretches complementary to (that is, stretches that could base pair with) the edited sequences. We found several that fit the model if we allowed G (guanine) residues to base pair with U (uridine) residues in addition to the C (cytosine) residues with which they more commonly pair (Blum et al., 1989). We then showed that the predicted gRNAs actually exist in *T. cruzi*, and that they consist of short RNA molecules, approximately 50 nucleotides in length, that have a nonencoded stretch of U residues at one end. The gRNAs form **duplex "anchors"** by hybridizing with (bonding to) specific mRNA sequences adjacent to the region to be edited and that contain additional A (adenine) and G residues, both of which can form base pairs with the U residues inserted by editing. Fully edited mRNA makes a perfect duplex with the gRNA. Thus, our newly discovered guide

BOX 6.1

Mitochondria.

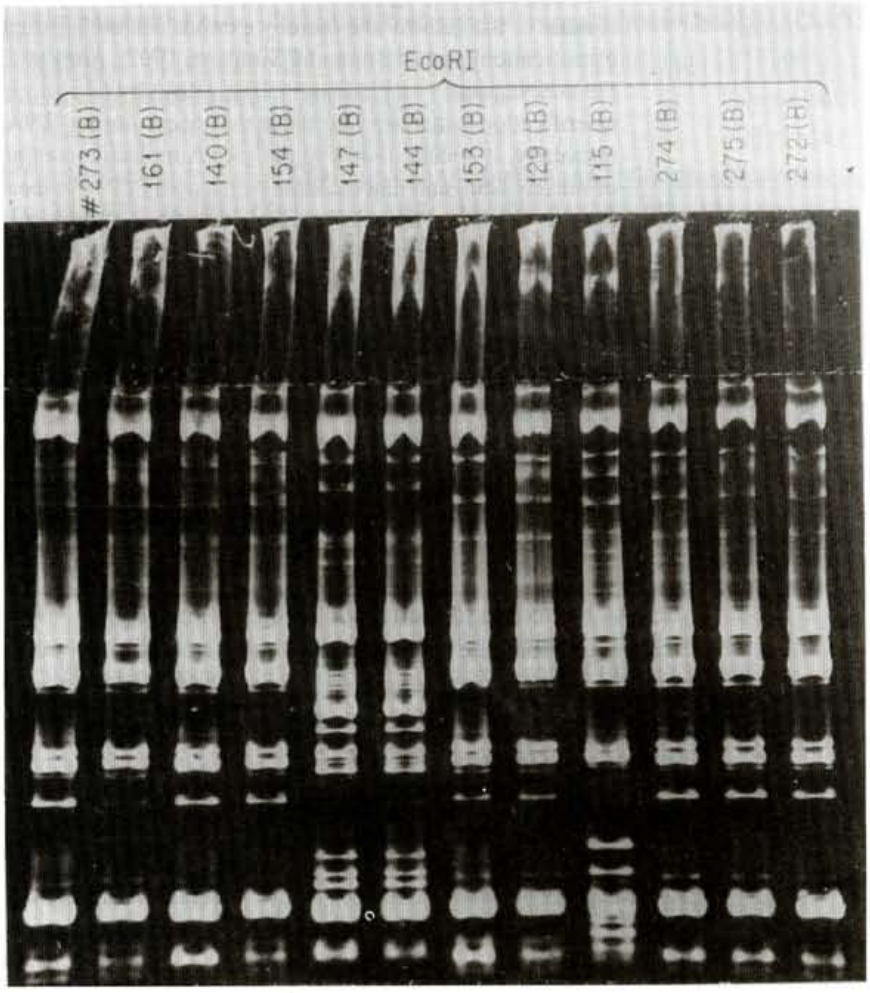
Mitochondria are the organelles in eukaryotic cells that produce chemical energy, stored in molecules of ATP (adenosine triphosphate), from the stepwise breakdown (oxidation) of foodstuffs such as glucose. They have two membranes, the inner one of which contains particular (cytochrome and flavoprotein) enzymes that make up the electron transport chain involved in energy production and the enzymes needed for synthesis of ATP. Within the matrix of the mitochondrion are located the enzymes of the TCA (tricarboxylic acid) cycle that converts pyruvate to acetyl Coenzyme-A and in so doing releases electrons that enter the cytochrome chain of the inner membrane. The matrix also contains the mitochondrial DNA, and mitochondrial ribosomes and associated translation machinery in which a small number of proteins is manufactured by using the information encoded in the DNA. The amount of genetic information encoded in animal and fungal mitochondrial DNA is variable, but is usually limited to that required for synthesis of cytochrome *b* and of several subunits each of cytochrome oxidase, NADH dehydrogenase, and ATP. Two ribosomal RNAs and a set of 24 transfer RNAs are also usually transcribed from the mitochondrial DNA, which in animal and fungal cells contains a sequence of information-containing nucleotides about 15 kb (kilobases) long. In higher plants, the amount of DNA in the mitochondria is much larger and their mitochondrial genomes contain many more genes.

FIGURE 6.10



Oswaldo Cruz Institute in Rio de Janeiro, Brazil, shown in a poster celebrating the first 90 years of the Fundacao Oswaldo Cruz. This "little castle," the oldest building in the Institute, is currently used for administration and the library.

FIGURE 6.11



Acrylamide gel electrophoresis of kinetoplast DNA from *Trypanosoma cruzi* isolated from several human patients. The *T. cruzi* parasites were isolated from human blood and grown in laboratory cultures, and the kDNA was isolated and digested with the EcoRI restriction enzyme and electrophoresed on an acrylamide gradient gel. The bands, which are made visible by staining with ethidium bromide, mainly represent fragments of minicircle DNA. The numbers refer to individual patients, all of whom share a common pattern of isoenzymes (B). Note the two distinct types of banding patterns, one group consisting of patients 147, 144, and 115, and the other group consisting of the remaining patients. (Reproduced, with permission, from Morel et al., 1980.)

BOX 6.2

Guide RNAs in flagellated protozoa.

The flagellated protozoa known as kinetoplastids contain a single mitochondrion that has a huge mass of mitochondrial DNA situated adjacent to the base of the flagellum. In trypanosomatids, a subgroup of kinetoplastids, this DNA consists of thousands of catenated minicircles and a smaller number of maxicircles all linked into a giant DNA network. The maxicircles are the evolutionarily related homologues of the mitochondrial DNA of human cells and contain the information for synthesis of two ribosomal ribonucleic acids (rRNAs) and several types of protein. The function of the minicircles was not understood until recently. Benne et al. (1986) and Shaw et al. (1988) discovered that several transcripts of the maxicircles are modified after transcription by the insertion and deletion of uridine residues within coding regions at multiple sites to form an "open reading frame" that encodes a protein. The extent of this RNA editing varies from insertion of four Us at three sites (which therefore offsets a "−1" frameshift) to insertion of hundreds of Us at hundreds of sites, creating an open reading frame apparently "from whole cloth" (Simpson et al., 1993). The information for this editing is contained in small RNAs, called guide RNAs (gRNAs) because they guide the editing process, which are transcribed both from maxicircle and from minicircle DNA molecules (Simpson et al., 1993). The 5′ ends of the gRNAs can form duplex "anchor" regions just downstream from the region to be edited (the region known as the PER), and the remainder of the molecule forms perfect duplexes with fully edited RNAs.

Two basic models have been proposed for the mechanism of the editing. The enzyme cascade model (Blum et al., 1990) proposes that an endonucleolytic cleavage occurs at the site of the first mismatch between the mRNA and the gRNA, the addition of a U to the 3′ hydroxyl group at the cleavage site, and a ligation (a bonding together) of the two resulting mRNA fragments. The added U bonds, by base pairing, with a guide A or G residue in the gRNA, and thereby extends the anchor duplex. The other model, the transesterification model (Blum and Simpson, 1992), proposes that the Us are transferred from the nonencoded 3′ oligo-U tail of the gRNA by two successive chemical reactions (transesterifications) that are like those that occur during RNA splicing. Whatever the precise chemistry, it is clear that a single gRNA molecule mediates the editing of a single block of nucleic acid, and that multiple overlapping gRNAs mediate the editing of multiple contiguous blocks (a "domain" of nucleic acid), as shown in Figure 6.12 for the MURF4 gene of the parasite *L. tarentolae*. In this lizard parasite, there are a limited number of gRNAs, each of which encodes a specific editing block. In contrast, in *Trypanosoma brucei* there are more than 1000 gRNAs that show extensive overlap even of their coding regions (Corell et al., 1993). The reason for this difference in the editing mechanism of the gRNAs in these two species of parasites is not clear, but it may be a result of changes that occurred during their evolutionary histories.

RNAs had come to the rescue of the central dogma. (We were pleased but also somewhat chagrined that the answer to the secret of editing was not something completely unexpected but was instead a novel system that still obeyed the simple rules of base pairing.) As discussed in Box 6.2, the precise mechanism by which the gRNAs mediate the insertion of U residues is still uncertain.

We then discovered that the genetic role of the previously mysterious minicircle kDNA molecules was to encode gRNAs, that is, the kDNAs contain the genetic information that results in formation of gRNA molecules (Sturm and Simpson, 1990; Pollard et al., 1990). A few gRNAs were encoded by the maxicircle DNA, but most were found to be encoded by the minicircles. In the lizard parasite *Leishmania tarentolae*, a trypanosomal parasite

related to *T. cruzi*, there is a single gRNA gene per minicircle, whereas in *T. cruzi* there are several gRNAs encoded in each minicircle (Avila and Simpson, unpublished results).

Editing (the insertion or deletion of uridine residues) was found to vary in different genes and in different kinetoplastid protozoal species from having a few Us inserted at a few sites to having hundreds of Us inserted at hundreds of sites. This latter, very extensive editing, termed **pan-editing**, involves multiple gRNAs that act sequentially in a complex process in which the first gRNA creates the anchor sequence for the second gRNA, the second makes the anchor for the third, and so forth (Maslov and Simpson, 1992). An example of a pan-edited cryptogene, together with the relevant overlapping gRNAs from *L. tarentolae*, is shown in Figure 6.12.

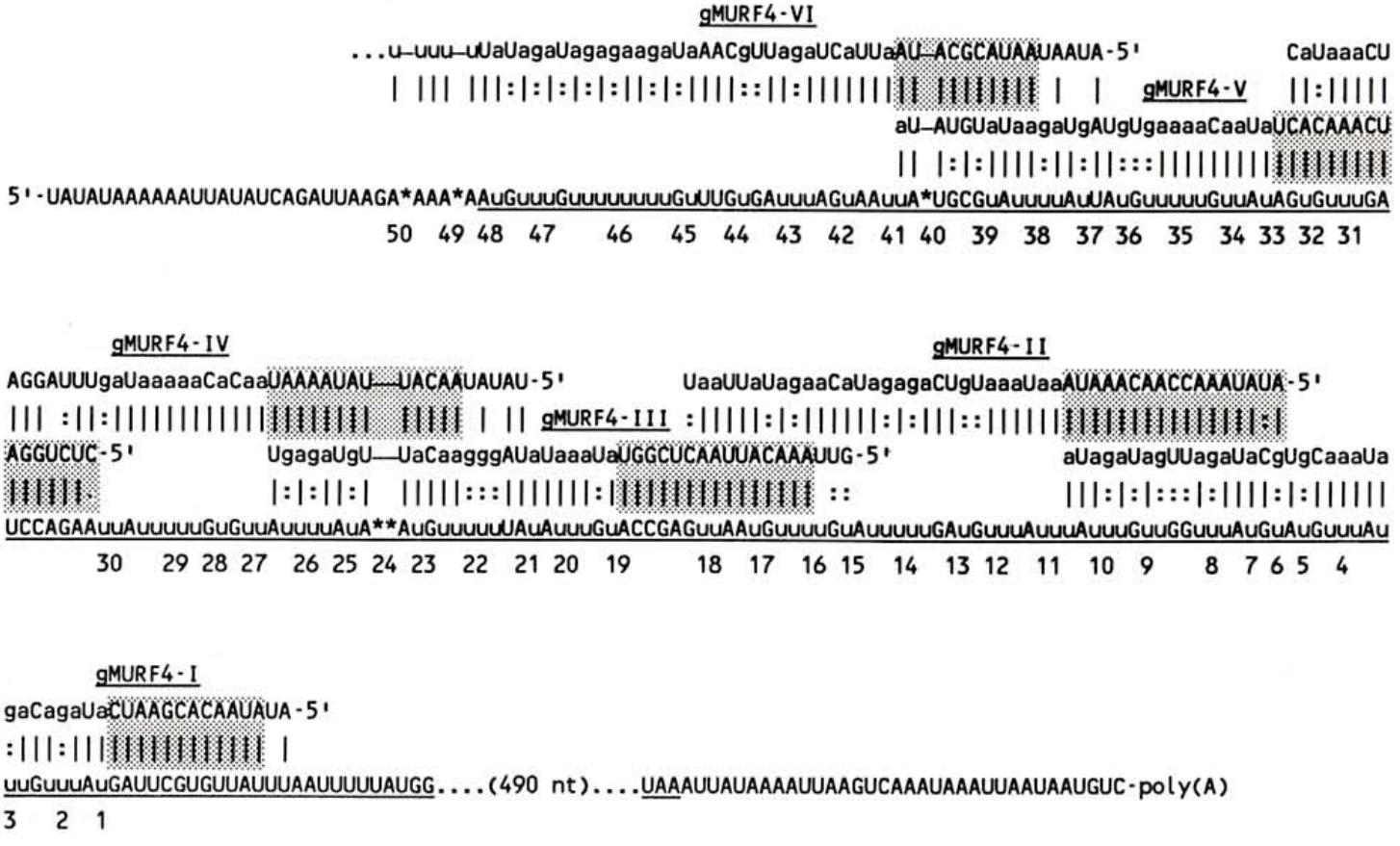


FIGURE 6.12

Pan-editing of transcripts from a maxicircle cryptogene of *Leishmania tarentolae*. The nucleotide sequence of the edited mRNA that encodes the information for protein synthesis (subdivided into three rows in the figure) is identified by the underlining. The uridines (u) inserted by editing increase the length of the original sequence encoded in the unedited gene by about twofold. The initial mRNA complimentary to the DNA of the gene is modified at 50 locations by RNA editing, as indicated by the numbers. The overlapping gRNAs (for example, "gMURF4-I"), which mediate the editing process, are shown above the sequence. Editing begins at gMURF4-I (lower left) and ends with gMURF4-VI. The gRNA/mRNA "anchor" duplex region is shaded for each editing block. The uridines inserted by editing are indicated by (u), and the guide nucleotides in the gRNAs by (a) or (g). Encoded (u) edited out of the mRNA are indicated by asterisks. G-C and A-U base pairs are shown as (|), and G-U base pairs as (:). (Reproduced, with permssion, from Maslov et al., 1992.)

MINICIRCLE kDNA AS A TARGET FOR DETECTION OF THE PARASITE

Because *T. cruzi* parasites occur in only small numbers in the blood of infected patients, they are difficult to detect. In the early 1980s, before the discovery of RNA editing and gRNAs, we reasoned that the kDNA minicircle, whatever its function, represented an appropriate molecular target for detection of this parasite by the technique of **hybridization**, because a large number of copies were present and they appeared to contain at least some short conserved sequences that would permit them to base pair with other similar molecules. In this technique (discussed also in Chapters 2 and 5), a radioactive single-stranded DNA fragment having a nucleotide sequence **complementary** to (able to base pair with) a short sequence on the minicircle, is allowed to form base pairs (that is, to hybridize) with kDNA molecules attached to a filter; the resultant "duplex" (double-stranded) molecules are detectable by their radioactivity.

Amplification of DNA: The Polymerase Chain Reaction

A chronically ill Chagas'-diseased patient has too few parasites for direct detection of parasite minicircle DNA by hybridization. However, at that time, Mullis and collaborators (Saiki et al., 1988) had just developed the **polymerase chain reaction (PCR)** technique by which small fragments of DNA are amplified (repeatedly replicated into enormous numbers of copies) in a test tube. This can be done if some nucleotide sequence information is already known. This technique has revolutionized molecular biology, and Mullis was awarded the Nobel Prize in 1993 for its discovery. Mullis claims that he got the idea for this novel technique while he was driving through the mountains of California with a female friend late one night, and he stopped the car to think it through (Mullis, 1990). Perhaps this proves the worth of leaving the lab once in a while and taking a drive with a friend!

The PCR technique depends on the ability to synthesize short DNA fragments of known nucleotide sequence and on the availability of a particular enzyme, **DNA polymerase**, which elongates a short DNA fragment in one direction by copying the nucleotide sequence from the other strand. In a short time, the method was improved by the use of a different DNA polymerase enzyme, one isolated from a high-temperature-adapted **thermophilic bacterium** that could survive repeated cycles of heating and cooling. The method is deceptively simple and consists of hybridizing two short synthetic single-stranded DNA molecules (called **primers**) onto the sequence to be amplified; adding new nucleotides to the 3′ end of each molecule by use of the heat-stable DNA polymerase; separating the strands by heating; and repeating this replication process many times (Figure 6.13). After 20 such cycles, the small DNA fragments terminated by the original primers will be amplified 2^{20}, that is, approximately one million times. As few as 10 molecules, even a single molecule, can be amplified and then detected by any of several hybridization-based methods, using either radioactivity or a nonradioisotopic visualization procedure. The main problem with this method is that it is too sensitive—any slight contamination of the reagents used can easily lead to amplification of the contaminating DNA rather than the DNA actually sought. Another problem is the occurrence of mutations, changes in the DNA nucleotides that may occur during the amplification process itself.

However, in spite of these problems, this method has led to a revolution in many fields of modern research. In the 1960s, for example, the only way to amplify DNA sequences was to insert them into bacteria, grow the bacteria through many generations, then extract

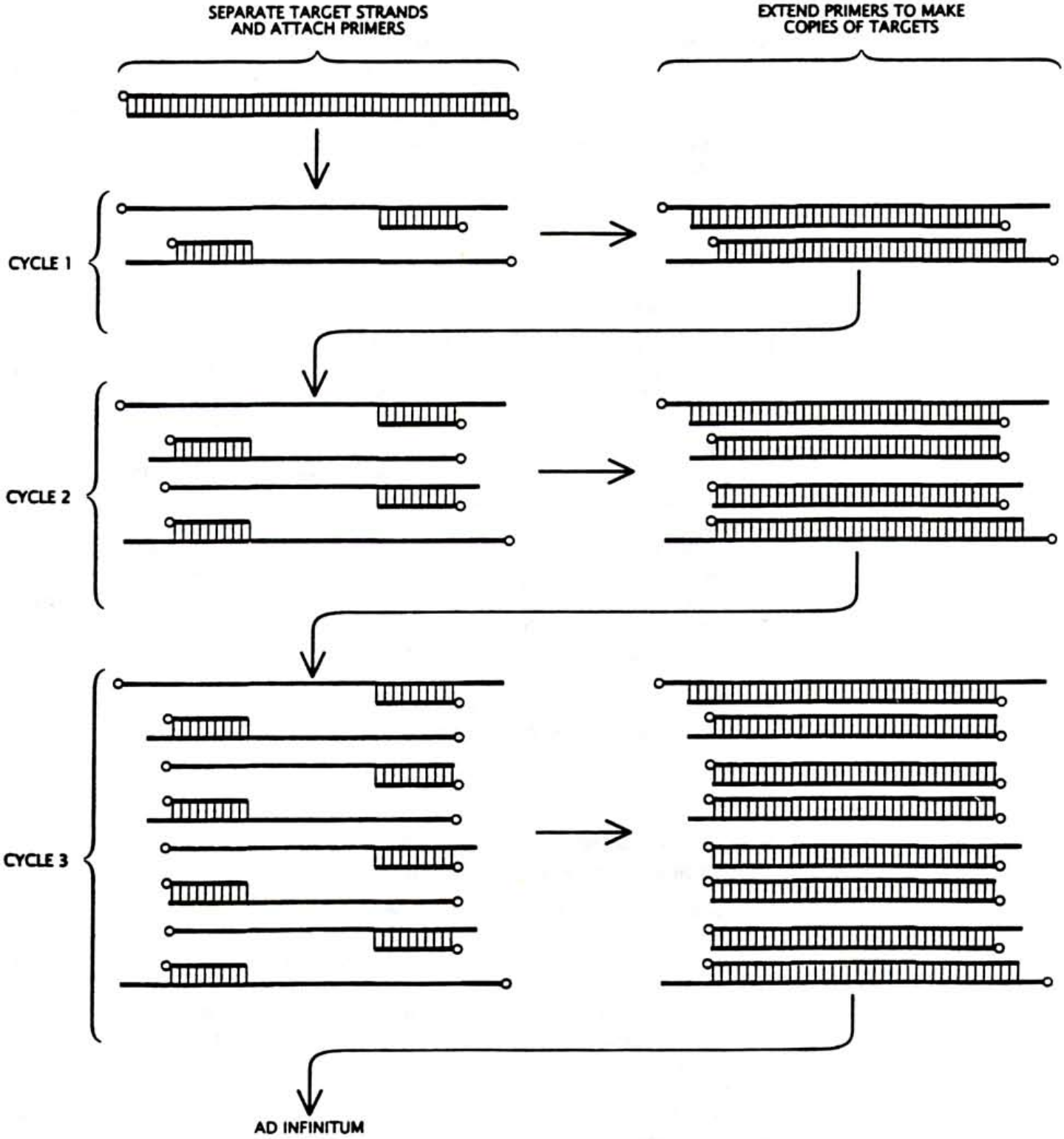


FIGURE 6.13

Polymerase chain reaction (PCR). The first three cycles of a PCR treatment are diagrammed, out of 20 to 30 such cycles that are normally carried out. The number of DNA molecules in the system is doubled with each cycle. The first step (upper left) is to separate the strands of double-stranded DNA by heating, and to then attach oligonucleotide primers to them. A heat-stable DNA polymerase is then used to extend the primers by adding nucleotides to one of their ends. (Reproduced, with permission, from Kary B. Mullis, The Unusual Origin of the Polymerase Chain Reaction. Copyright © 1990 by Scientific American, Inc. All rights reserved.)

the DNA they had produced. Now, a DNA sequence can be amplified rapidly without using bacteria at all—just enzymes in a test tube! As trumpeted in newspapers and magazines, PCR has come to play a major role in forensic science, in which the presence of a particular individual at the scene of a crime can be demonstrated by the amplification of distinctive nucleotide sequences from the DNA that occurs in a single hair follicle or in a minute spot of blood or some other biological material (Blake et al., 1992). In paleontology (discussed also in Chapter 3), workers have been able to amplify DNA sequences from fossils millions of years old for direct comparison of their DNA with that of their living relatives.

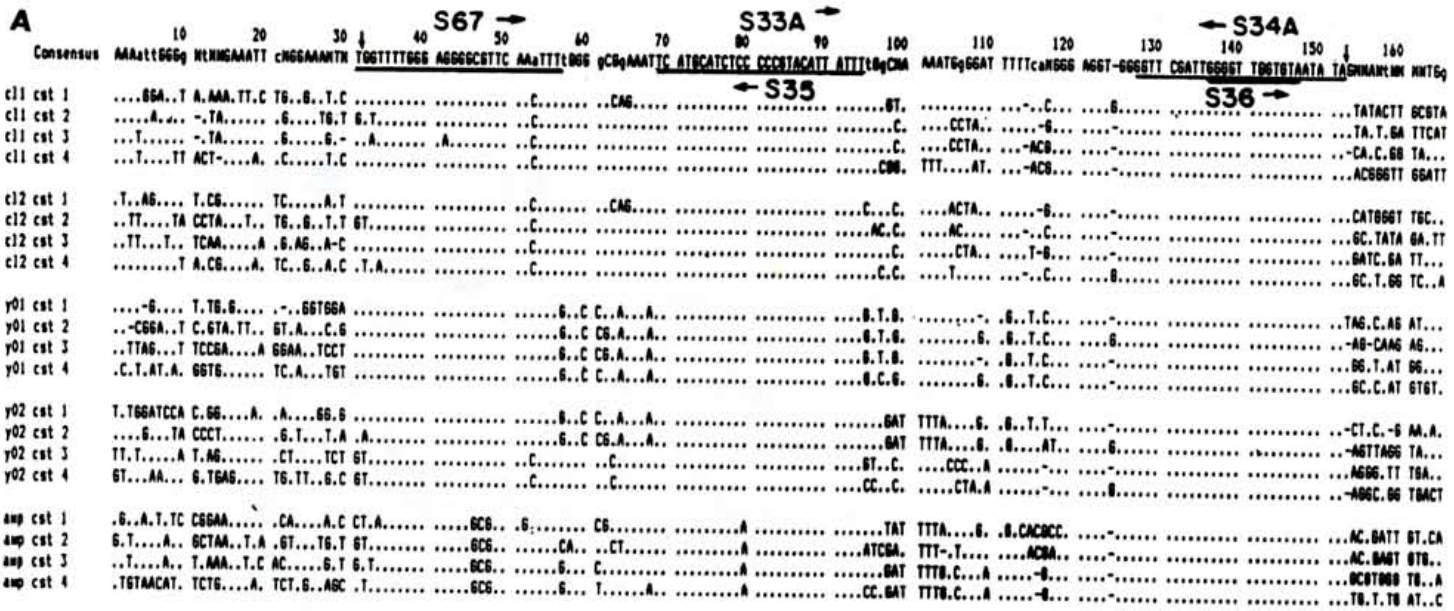
Ancient DNA

The first ancient DNA was amplified in 1984 from a museum specimen of an animal called a quagga, a relatively recently extinct horse-like creature (Higuchi et al., 1984). A fragment of the mitochondrial genome of this specimen was PCR-amplified and sequenced. Soon thereafter, DNA fragments were amplified from 2,400-year-old Egyptian mummies (Pääbo, 1985) and 5,500-year-old human bone (Hanni et al., 1990). These discoveries were soon surpassed by amplification of DNA fragments from 18-million-year-old *Magnolia* leaves (Golenberg et al., 1990), 25- to 30-million-year-old fossil termites in amber (DeSalle et al., 1992), and recently, 120- to 135-million-year-old fossil weevils (Cano et al., 1993), also preserved in amber. There are, of course, cautionary notes being sounded, as discussed in Chapter 3, and it is possible that some of these reports may represent amplification of contaminating DNA from modern organisms, a well-known problem of PCR (Pääbo, 1989). It has also been argued that it is theoretically implausible for the chemical structure of DNA to survive for millions of years without being completely degraded (Lindahl, 1993). The evidence, however, seems strong that a number of these sequences actually have been obtained from fossilized DNA, because phylogenetic reconstructions derived from these data agree well with the known paleontological identity of the fossils.

PCR Amplification of *Trypanosoma cruzi* kDNA: The Chilean Mummy Connection

My interest in applying PCR to the detection of *T. cruzi* in biological materials was initially stimulated by a phone call from Christian Orrega, who was working in the laboratory of Alan Wilson, a pioneer in the amplification of ancient DNA (Pääbo et al., 1988). Orrega had access to tissues from 2,500-year-old desiccated mummies from Chile. The tissues reportedly showed at autopsy examination (now long delayed!) observable manifestations of Chagas' disease (Rothhammer et al., 1985). Orrega asked whether I would develop a PCR-based method to detect *T. cruzi* in such ancient tissue if he supplied me with tissue samples. Intrigued, I agreed to the project.

To use the PCR technique for amplifying parasite DNA, we first had to find appropriately conserved (that is, evolutionarily little changed) sequences with which the short PCR **oligonucleotide** primers could hybridize. Minicircle molecules were cloned and sequenced from three different strains of *T. cruzi*, and we found that the 1,500 base pair (bp) molecules were organized into four conserved and four variable regions, as shown in Figure 6.14 (Degrave et al., 1988). The conserved regions in other kinetoplastid protozoal species were shown to be sequences involved in DNA replication, and the variable regions, at least in minicircle molecules from the lizard parasite, *L. tarentolae*, and the African pathogenic trypanosome, *T. brucei*, to encode gRNA genes (Sturm and Simpson, 1991; Sturm and Simpson, 1990). When we aligned the sequences of 20 regions from five



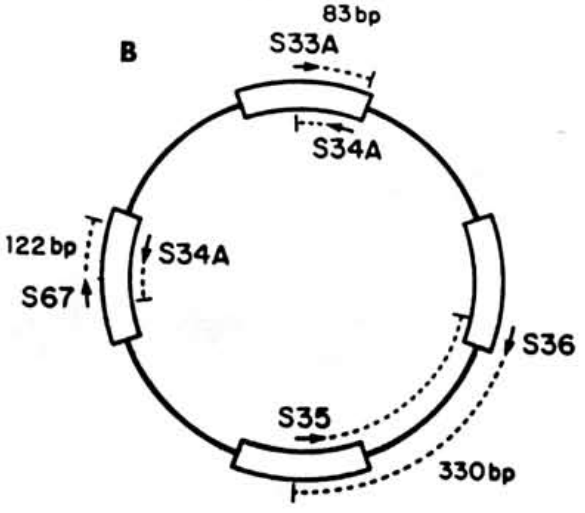
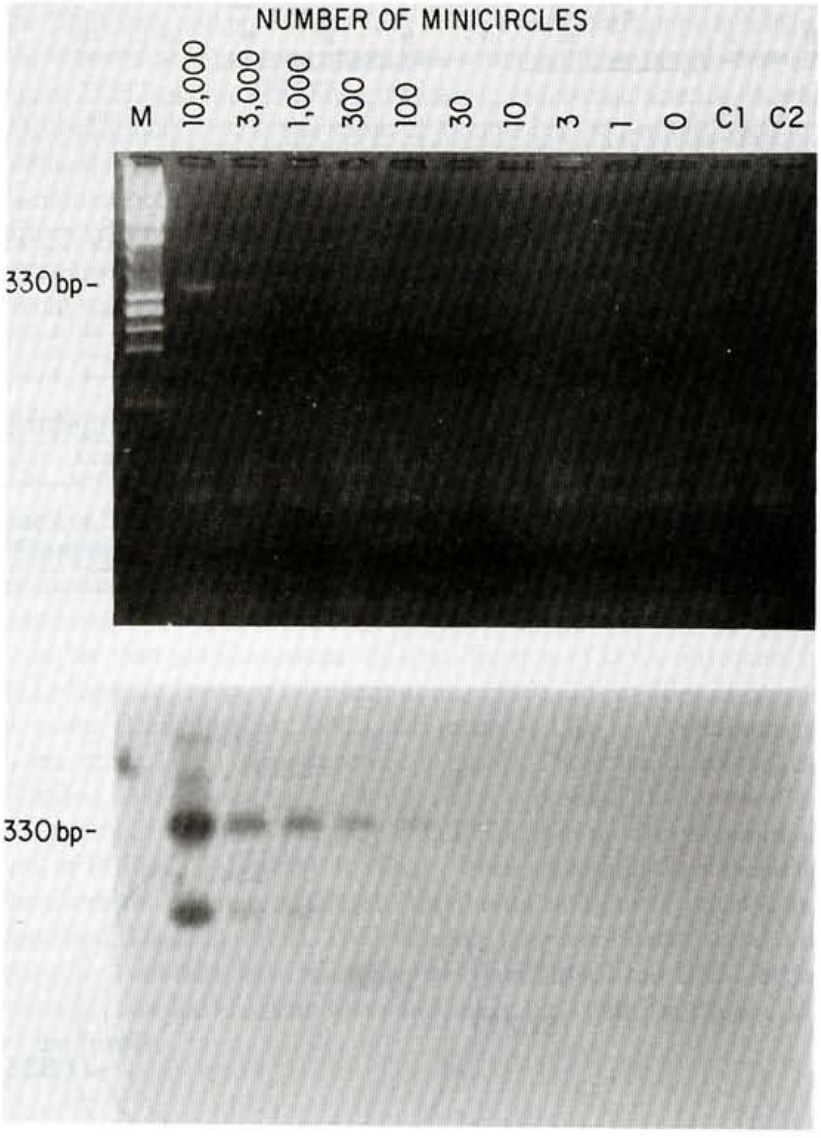


FIGURE 6.14

Alignment of conserved regions in five minicircles from three strains of *Trypanosoma cruzi*. Part A: Alignment of sequences showing highly conserved and less conserved regions. The locations of the primers (for example, "S34A") used for PCR are indicated by underlining, and their polarity by arrows. Dots indicate that identical nucleotides occur in all sequences; gaps are indicated by (-). The consensus sequence (listed at the top of the figure) shows well-conserved nucleotides as A, C, G, T, and less-conserved nucleotides as M, N, and a, c, g, and t. Part B: The circular diagram shows a minicircle having four conserved regions (indicated by boxes) and the localization of primers used for PCR. To simplify the diagram, only a single PCR product is shown for each conserved region.

sets of minicircles, we identified several highly conserved sequences to serve as primers for PCR amplification, either of 80 bp or 120 bp fragments within the conserved region, or of 330 bp fragments containing the adjacent variable regions (Figure 6.14). As summarized in Figure 6.15, we showed that this method worked and was specific for as few as 10 to 100 minicircle molecules from *T. cruzi* (Sturm et al., 1989), and that the method

FIGURE 6.15



Sensitivity of the PCR amplification of *Trypanosoma cruzi* minicircle DNA. kDNA was digested in guanidine blood lysate with a chemical nuclease (orthophenanthraline-Cu++) and diluted with kDNA-free lysate to obtain known numbers of molecules as a substrate for each amplification. The upper panel shows that after staining the DNA with a fluorescent dye (ethidium bromide), a minimum of 10,000 minicircles can be detected. With a more sensitive technique for visualizing DNA (the Southern blot method), as few as 30 minicircles can be detected. The lower panel shows an autoradiograph of a Southern blot of this gel hybridized with a radioactive oligonucleotide probe for the minicircle conserved region. The identity of the rapidly migrating band seen in the lower panel in the vertically oriented lanes labeled 10,000, 3,000, and 1,000 is not known. Lanes C1 and C2 (at right) show results obtained for negative control amplifications, carried out without the DNA substrate, to check for contamination of reagents. (Reproduced, with permission, from Avila et al., 1991.)

was unaffected by a several billionfold excess of human DNA (Figure 6.16). We then showed that amplification of these fragments could be accomplished for minicircles extracted from numerous strains of *T. cruzi* isolated from diverse regions throughout Latin America (Avila et al., 1991).

Diagnosis of Chagas' Disease in Patients by PCR Amplification of Minicircle kDNA

At this point, this method seemed more appropriate to detect *T. cruzi* parasites in live chronically ill patients, rather than in Chilean mummy tissues. It would be important to know whether live parasites of known strains were circulating in chronic Chagasic patients, or whether the disease was some sort of autoimmune phenomenon that persisted long after the parasites had disappeared.

To apply the PCR method to detect parasite DNA in patients, we needed a method to isolate parasite DNA from human blood. This presented a problem because blood contains a substance that inhibits PCR; we would have to use relatively large quantities (5 to 10 ml) of blood to detect the low numbers of parasites expected to be circulating in the

veins of chronically ill patients. Herbert Avila, a graduate student in my laboratory, found that mixing blood with an equal volume of a particular concentrated salt solution (5 M guanidine-0.2 M ethylenediaminetetraacetic acid (EDTA); Avila et al., 1992) would **lyse** (disrupt and solubilize) the parasites as well as the host blood cells, releasing their DNA molecules into the medium, and would preserve the DNA against degradation even at or above room temperature for several weeks (Figure 6.17). This was an important breakthrough because samples could thus be taken in the field and transported to the laboratory without refrigeration.

Because of the unique molecular organization of the parasite kinetoplast DNA, a method had to be found to release the catenated (interlocked) minicircles from the network so that the sensitivity of the procedure could be increased. Otherwise, a single network of DNA from a single trypanosome in even a 10 to 20 ml blood sample would inevitably be lost

FIGURE 6.16

Lack of effect of several billion-fold excess of human DNA on amplification of the 122 bp *Trypanosoma cruzi* minicircle fragment. The number of PCR cycles is indicated above each vertically oriented lane. The upper panel shows the stained gel, and the lower panel the autoradiograph of a Southern blot of this gel hybridized with a radioactive probe for the minicircle conserved region (see Figure 6.5). Primer controls (upper right) are control samples to which no DNA has been added. (Reproduced, with permission, from Sturm et al., 1989.)

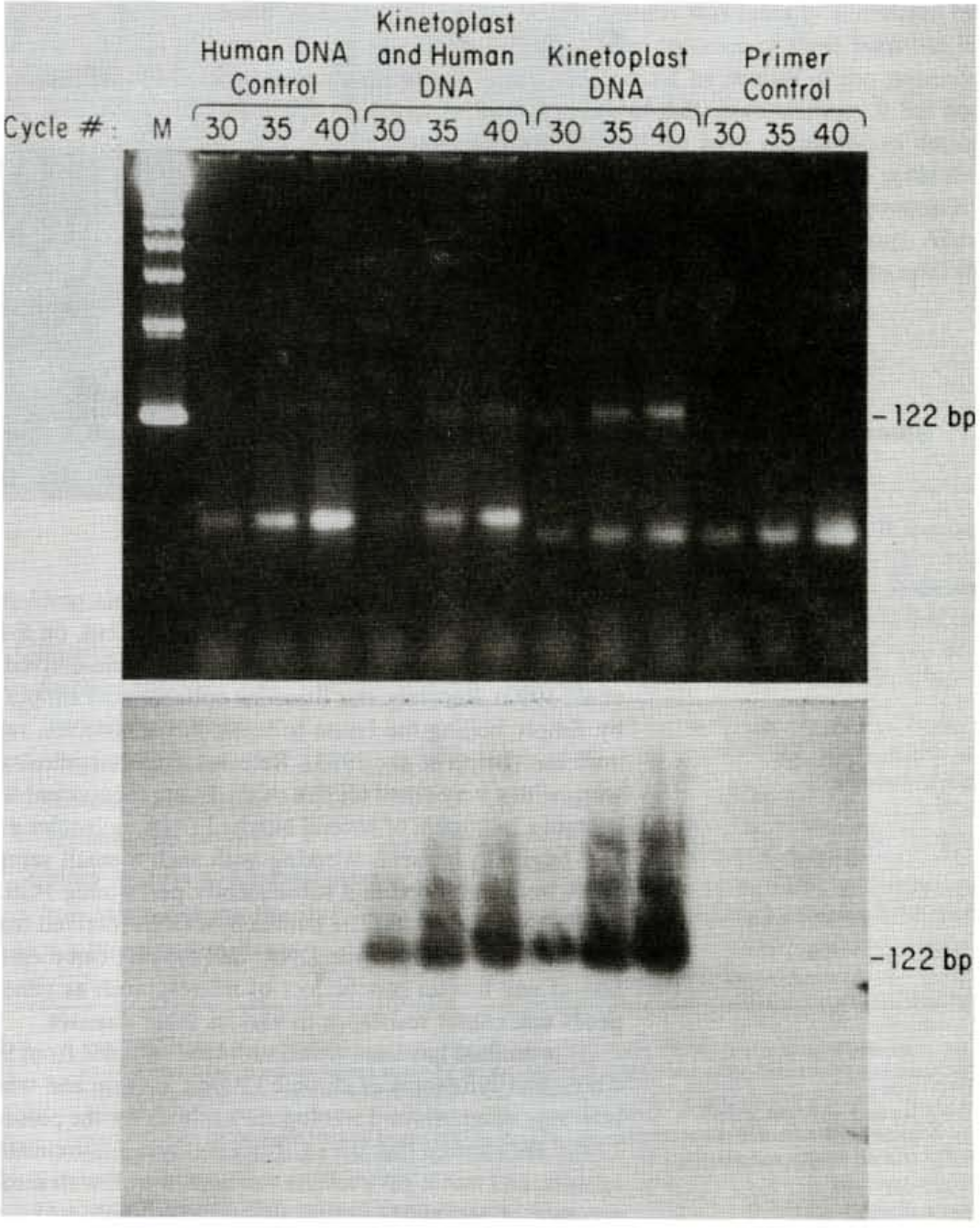
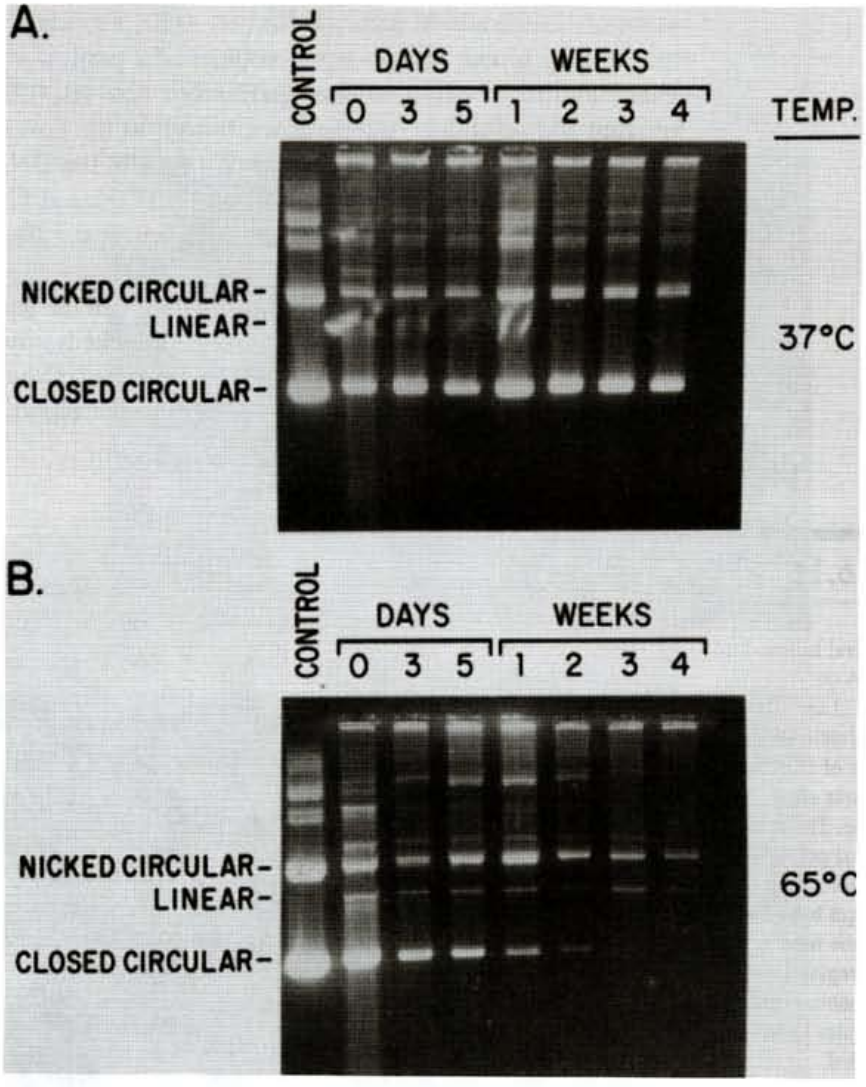


FIGURE 6.17

Thermal stability of closed circular DNA in the guanidine blood lysate at two temperatures. The positions of three types of "Bluescript plasmid" DNA molecules (closed circular, nicked circular, and linear) are shown. Note that closed circular DNA is stable for several weeks at 37°C. (Reproduced, with permission, from Avila et al., 1991.)



during the DNA isolation process. To solve this problem, we used a chemical **nuclease** (in particular, orthophenanthraline-Cu++) to cut, on average, each minicircle molecule once and thereby release 10,000 linear minicircle-derived molecules into the lysate (Avila et al., 1992). Recently, our Brazilian collaborators simplified and improved this procedure by simply boiling the lysate to break the minicircles, rather than by using the chemical nuclease (Britto et al., 1993). Release of the minicircles from the network allows us to work with a very small aliquot of the lysate (equivalent to only a few drops of blood) that contain a minimum of several hundred DNA molecules even if only a single trypanosome was originally present. Working with such a small sample facilitates isolating the total DNA from the blood and subsequently performing PCR amplification of parasite minicircle DNA fragments. The human host DNA (derived from white blood cells) is also isolated together with parasite DNA, and the mix can therefore be used for PCR amplification of any human genetic loci of interest, such as genes that cause genetic diseases or genes that confer resistance to various other diseases.

This method has been tested on blood samples from 95 patients from Brazil, many of whom had symptoms of chronic Chagas' disease and who had been previously tested by both xenodiagnosis and serological methods for the presence of the infection (Avila et al., 1993). We showed that the PCR method was approximately twice as sensitive as xenodiagnosis, and that it gave results that agreed well with serological assays that measured the presence of antibodies against the parasite (Table 6.1).

Amplification of the 330 bp variable region fragment of minicircle DNA yields one additional piece of information. Because these fragments are derived from all the minicircles in the network, and because the nucleotide sequences in these minicircle variable regions differ between different parasite schizodemes, the amplified DNA permits the specific strain of parasite present in a patient to be classified into an identified group. This may prove important, because different strains of the parasites may cause different clinical disease syndromes and may be susceptible to treatment with differing drugs. Such groupings should also allow us to learn which strains of the parasite are present in patients, insects, and other animals from differing geographical regions.

A MODEST PROPOSAL

Our scientific odyssey began with Charles Darwin's mysterious disease and continued with our recent research on *T. cruzi* and PCR, which actually was initially stimulated by a request by a colleague to examine the possibility of the presence of these parasites in 4000-year-old Chilean mummies, but which was sidetracked by studies of the presence of parasites in chronically ill Chagasic patients. In spite of these meanderings, it has not escaped our attention that the PCR amplification method we have developed to detect small numbers of parasites in chronic Chagasic patients might in fact be used to settle the question of Darwin's illness. I would like to end this chapter with a "modest proposal," namely, that we remove a sample of Charles Darwin for PCR analysis of *T. cruzi*. After all, he was interred in a very accessible location—in Westminster Abbey, right next to Isaac Newton—and, we only need a little piece!

TABLE 6.1

Comparison of results for blood samples analyzed by serologic, xenodiagnostic, and PCR tests for Chagas' disease.*

Sample Type	Number of Samples	Number of PCR Results	
		Positive	Negative
Clinically diagnosed Chagas' disease and			
Serology positive	91	91	0
Serology negative	1	1	0
Serology positive and			
Xenodiagnosis positive	48	48	0
Xenodiagnosis negative	35	35	0
Serology-negative nonchagasic patients			
from Virgin de Lapa, Brazil	3	2	1
UCLA Blood Bank donors	18	0	18

*The Chagasic samples are from patients from Virgin de Lapa, Minas Gerais, Brazil. The serological tests represent several standard immunological assays for the presence of antibodies to *T. cruzi*. Note that 35 patients who were xenodiagnosis-negative but serologically positive also proved positive by PCR, indicating that PCR was approximately twice as effective in detecting parasites as xenodiagnosis. In addition, two of the three patients who were serologically negative proved positive by PCR, suggesting either that PCR was more efficient than serology or that these were false-positive PCRs. All 18 University of California, Los Angeles (UCLA) blood bank donors proved negative by PCR.

Source: Reproduced, with permission, from Avila et al., 1993.

REFERENCES

Adler, D. 1989. Darwin's illness. *Isr. J. Med. Sci. 25:* 218–221.

Adler, S. 1959. Darwin's illness. *Nature 184:* 1102–1103.

Avila, H., Goncalves, A.M., Nehme, N.S., Morel, C.M., and Simpson, L. 1991. Schizodeme analysis of *Trypanosoma cruzi* stocks from South and Central America by analysis of PCR-amplified minicircle variable region sequences. *Mol. Biochem. Parasitol. 42:* 175–188.

Avila, H.A., Sigman, D.S., Cohen, L.M., Millikan, R.C., and Simpson, L. 1992. Polymerase chain reaction amplification of *Trypanosoma cruzi* kinetoplast minicircle DNA isolated from whole blood lysates: Diagnosis of chronic Chagas' disease. *Mol. Biochem. Parasitol. 48:* 211–222.

Avila, H.A., Pereira, J.B., Thiemann, O., De Paiva, E., Degrave, W., Morel, C.M., and Simpson, L. 1993. Detection of *Trypanosoma cruzi* in blood specimens of chronic chagasic patients by polymerase chain reaction amplification of kinetoplast minicircle DNA: Comparison with serology and xenodiagnosis. *J. Clin. Microbiol. 31:* 2421–2426.

Benne, R., Van den Burg, J., Brakenhoff, J., Sloof, P., Van Boom, J., and Tromp, M. 1986. Major transcript of the frameshifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell 46:* 819–826.

Bernstein, R.E. 1984. Darwin's illness: Chagas' disease resurgens. *J. Roy. Soc. Med. 77:* 608–609.

Blake, E., Mihalovich, J., Higuchi, R., Walsh, P.S., and Erlich, H. 1992. Polymerase chain reaction (PCR) amplification and human leukocyte antigen (HLA)-DQ alpha oligonucleotide typing on biological evidence samples: Casework experience. *J. Forensic Sci. 37:* 700–726.

Blum, B., Bakalara, N., and Simpson, L. 1990. A model for RNA editing in kinetoplastid mitochondria: "Guide" RNA molecules transcribed from maxicircle DNA provide the edited information. *Cell 60:* 189–198.

Blum, B., and Simpson, L. 1992. Formation of gRNA/mRNA chimeric molecules *in vitro*, the initial step of RNA editing, is dependent on an anchor sequence. *Proc. Natl. Acad. Sci. USA 89:* 11944–11948.

Britto, C., Cardoso, M.A., Wincker, P., and Morel, C.M. 1993. A simple procedure for the physical cleavage of *Trypanosoma cruzi* kinetoplast DNA present in blood samples and its use on PCR-based diagnosis of chronic Chagas disease. *Mem. Inst. Oswaldo Cruz 88:* 171–172.

Cano, R.J., Poinar, H.N., Pieniazek, N.J., Acra, A., and Poinar, G. O., Jr. 1993. Amplification and sequencing of DNA from a 120- to 135-million-year-old weevil. *Nature 363:* 536–538.

Cech, T. R. 1991. RNA editing: World's smallest introns. *Cell 64*: 667–669.

Corell, R.A., Feagin, J.E., Riley, G.R., Strickland, T., Guderian, J.A., Myler, P.J., and Stuart, K. 1993. *Trypanosoma brucei* minicircles encode multiple guide RNAs which can direct editing of extensively overlapping sequences. *Nucleic Acids Res. 21:* 4313–4320.

Degrave, W., Fragoso, S., Britto, C., Van Heuverswyn, H., Kidane, G., Cardoso, M., Mueller, R., Simpson, L., and Morel, C. 1988. Peculiar sequence organization of kinetoplast DNA minicircles form *Trypanosoma cruzi*. *Mol. Biochem. Parasitol. 27:* 63–70.

DeSalle, R., Gatesy, J., Wheeler, W., and Grimaldi, D. 1992. DNA sequences from a fossil termite in Oligo-Miocene amber and their phylogenetic implications. *Science 257:* 1933–1936.

Feagin, J.E., Shaw, J.M., Simpson, L., and Stuart, K. 1988. Creation of AUG initiation codons by addition of uridines within cytochrome b transcripts of kinetoplastids. *Proc. Natl. Acad. Sci. USA 85:* 539–543.

Goldstein, J.H. 1989. Darwin, Chagas' disease, mind, and body. *Perspec. Biol. Med. 32:* 586–601.

Golenberg, E. M., Giannasi, D.E., Clegg, M.T., Smiley, C.J., Durbin, M., Henderson, D., and Zurawski, G. 1990. Chloroplast DNA sequence from a miocene Magnolia species. *Nature 344:* 656–658.

Hanni, C., Laudet, V., Sakka, M., Begue, A., and Stehelin, D. 1990. Amplification of mitochon-

drial DNA fragments from ancient human teeth and bones. *C. R. Acad. Sci. (Paris) III. 310:* 365–370.

Higuchi, R., Bowman, B., Freiberger, M., Ryder, O.A., and Wilson, A. C. 1984. DNA sequences from the quagga, an extinct member of the horse family. *Nature 312:* 282–284.

Kirchoff, L.V., Gam, A.A., and Gillan, F.C. 1987. American trypanosomiasis (Chagas' disease) in Central American immigrants. *Am. J. Med. 82:* 915–920.

Kirchoff, L.V. 1989. Is *Trypanosoma cruzi* a new threat to our blood supply? *Ann. Intern. Med. 111:* 773–775.

Lindahl, T. 1993. Instability and decay of the primary structure of DNA. *Nature 362:* 709–715.

Maslov, D.A., and Simpson, L. 1992. The polarity of editing within a multiple gRNA-mediated domain is due to the formation of anchors for upstream gRNAs by downstream editing. *Cell 70:* 459–467.

Morel, C., Chiari, E., Camargo, E., Mattei, D., Romanha, A., and Simpson, L. 1980. Strains and clones of *Trypanosoma cruzi* can be characterized by restriction endonuclease fingerprinting of kinetoplast DNA minicircles. *Proc. Natl. Acad. Sci. USA 77:* 6810–6814.

Mullis, K.B. 1990. The unusual origin of the polymerase chain reaction. *Scientific American 262:* 56–65.

Pääbo, S. 1985. Molecular cloning of ancient Egyptian mummy DNA. *Nature 314:* 644–645.

Pääbo, S. 1989. Ancient DNA: Extraction, characterization, molecular cloning, and enzymatic amplification. *Proc. Natl. Acad. Sci. USA 86:* 1939–1943.

Pääbo, S., Gifford, J.A., and Wilson, A.C. 1988. Mitochondrial DNA sequences from a 7000-year old brain. *Nucleic Acids. Res. 16:* 9775–9787.

Pollard, V.W., Rohrer, S.P., Michelotti, E.F., Hancock, K., and Hajduk, S.L. 1990. Organization of minicircle genes for guide RNAs in *Trypanosoma brucei. Cell 63:* 783–790.

Prata, A. 1981. *Carlos Chagas—Coletanea de Trabalhos Cientificos.* Editora Universidade de Brasilia, Brasilia.

Rothhammer, F., Allison, M.J., Nunez, L., Standen, V., and Arriaza, B. 1985. Chagas' disease in pre-Columbian South America. *Amer. J. Phys. Anthropol. 68:* 495–498.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K., and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science 239:* 487–491.

Schmidtmeyer, P., 1824. *Travels into Chile.* (London: Longman, Hurst, Rees, Orme, Brown and Green).

Schmunis, G.A. 1991. *Trypanosoma cruzi*, the etiologic agent of Chagas' disease: Status in the blood supply in endemic and nonendemic countries. *Transfusion 31:* 547–557.

Shaw, J., Feagin, J.E., Stuart, K., and Simpson, L. 1988. Editing of mitochondrial mRNAs by uridine addition and deletion generates conserved amino acid sequences and AUG initiation codons. *Cell 53:* 401–411.

Simpson, L. 1972. The kinetoplast of the hemoflagellates. *Int. Rev. Cytol. 32:* 139–207.

Simpson, L. 1987. The mitochondrial genome of kinetoplastid protozoa: Genomic organization, transcription, replication and evolution. *Ann. Rev. Microbiol. 41:* 363–382.

Simpson, L., Maslov, D.A., and Blum, B. 1993. RNA editing in *Leishmania* mitochondria. In: R. Benne (Ed.), *RNA Editing* (New York: Ellis Horwood), pp. 53–85.

Stuart, K. 1993. RNA editing—the alteration of protein coding sequences of RNA. In: R. Benne (Ed.), *RNA Editing* (New York: Ellis Horwood), pp. 25–52.

Sturm, N.R., Degrave, W., Morel, C., and Simpson, L. 1989. Sensitive detection and schizodeme classification of *Trypanosoma cruzi* cells by amplification of kinetoplast minicircle DNA sequences: Use in diagnosis of Chagas Disease. *Mol. Biochem. Parasitol. 33:* 205–214.

Sturm, N.R., and Simpson, L. 1990. Kinetoplast DNA minicircles encode guide RNAs for editing of cytochrome oxidase subunit III mRNA. *Cell 61:* 879–884.

Sturm, N.R., and Simpson, L. 1991. *Leishmania tarentolae* minicircles of different sequence classes

encode single guide RNAs located in the variable region approximately 150 bp from the conserved region. *Nucleic Acids Res. 19:* 6277–6281.

Thielen, J. 1991. *Science Heading for the Backwoods, Images of the Expeditions Conducted by the Oswaldo Cruz Institute to the Brazilian Hinterland 1911/1913.* (Rio de Janeiro: Fundaçao Oswaldo Cruz).

Tibayrenc, M., Ward, P., Moya, A., and Ayala, F. 1986. Natural populations of *Trypanosoma cruzi*, the agent of Chagas' disease, have a complex multiclonal structure. *Proc. Natl. Acad. Sci. USA 83:* 115–119.

Tibayrenc, M., Kjellberg, F., and Ayala, F.J. 1990. A clonal theory of parasitic protozoa: The population structures of *Entamoeba, Giardia, Leishmania, Naegleria, Plasmodium, Trichomonas,* and *Trypanosoma* and their medical and taxonomical consequences. *Proc. Natl. Acad. Sci. USA 87:* 2414–2418.