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Heritable Integration of kDNA Minicircle Sequences from *Trypanosoma cruzi* into the Avian Genome: Insights into Human Chagas Disease

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Summary

We demonstrate the genetic transfer of DNA between eukaryotes from different kingdoms. The mitochondrial kinetoplast DNA (kDNA) of the intracellular parasite *Trypanosoma cruzi* is transferred to human patients with Chagas disease. This transfer was reproduced experimentally in rabbits and chickens. The kDNA is integrated into the host genome. In the human chromosomes, five loci were identified as integration sites, and the β-globin locus and LINE-1 retrotransposons were frequently targeted. Short repeated sequences in the parasite and the target host DNAs favor kDNA integration by homologous recombination. Reproduced kDNA was present in offspring of chronically infected rabbits and in chickens hatched from *T. cruzi*-inoculated eggs. kDNA incorporated into the chicken germline was inherited through the F2 generation, in the absence of persistent infection. kDNA integration represents a potential cause for the autoimmune response that develops in a percentage of chronic Chagas patients, which can now be approached experimentally.

Introduction

During his travels, Charles Darwin entered the Province of Mendoza in Argentina, where he was preyed upon by a *benchuca*, the triatomine bug of the Pampas. This bug, which is now known to be a vector of Chagas disease, rapidly inserts its proboscis and drinks its fill of blood in less than 10 min, transforming its abdomen from a flat wafer to a globular form. From this historical report (Darwin, 1835), the future impact of the insect and resulting disease could not be foreseen.

Carlos Chagas described the *Trypanosoma cruzi* epimastigote form in the gut of the triatomine bug *Panstrongylus megistus* (*Hemiptera: Reduviidae*) that commonly infested the primitive huts of the poorer inhabitants of the State of Minas Gerais, Brazil (Chagas, 1909). Soon after, he found flagellated *T. cruzi* trypomastigote forms in the blood of a cat and of a child, traced the development of *T. cruzi* in its vertebrate and invertebrate hosts, and made the initial report of this disease (Chagas, 1911), which now bears his name. Vianna (1911) showed that this trypanosome reproduced intracellularly in a round amastigote form by binary fission in various tissues of the mammalian host. Mammals belonging to several classes (marsupials, edentates, lagomorphs, rodents, carnivores, primates, and chiropterans) are hosts for *T. cruzi*. Birds are refractory to *T. cruzi* infection (reviewed in Teixeira [1987]).

American trypanosomiasis infections span an extensive geographical area between 42°N in the United States to 43°S in Argentina. Human Chagas disease is considered the most significant parasitic disease in Latin America. It is estimated that 16–18 million people are infected by *T. cruzi*. As a consequence, approximately 50,000 deaths occur every year. The acute infection usually goes unrecognized and enters a chronic stage that persists throughout the host's life span. However, roughly 30% of infected individuals eventually will develop disease with an array of possible manifestations affecting the heart, the digestive tract, and/or the peripheral nervous system (Prata, 2001).

The mitochondrion of *T. cruzi* contains the largest amount of extranuclear genetic material of any eukaryote. This kinetoplast DNA (kDNA) contains a few dozen maxicircles (23 kb) and several thousand minicircles (1.4 kb) catenated into a large and complex network (Klingbeil et al., 2002), comprising 10%–15% of the total cell DNA (Lukes et al. 2002). The maxicircles are the functional equivalent of our mitochondrial DNA but contain several genes that require substantial posttranscriptional modification by RNA editing (Estevez and Simpson, 1999). The minicircles encode the guide RNAs that direct this process, contributing to the enormous content and heterogeneity in the mitochondrial DNA component of these organisms (Campbell et al., 2003).

Perhaps the most important problem in the field of Chagas disease research is determination of the pathogenesis of the chronic disease. Various hypotheses have been proposed, ranging from degradation of the affected tissues over time caused directly by the presence of the parasite to the possibility of an autoimmune response that is not dependent on the persistence of live infection (Teixeira et al., 1996). Our previous studies have indicated that kDNA integration into the vertebrate host genome occurs frequently (Teixeira et al., 1994). In this report, we document numerous instances of kDNA integration into the genomes of patients infected with *T. cruzi*. Furthermore, we demonstrate that this integration can occur in the experimentally accessible rabbit model system and that kDNA integration can be perpetuated through the germline in chickens in the absence of infection. The possibility of genomic damage inflicted upon the host by the horizontal DNA transfer from this parasite may hold the key to the differential activation of chronic Chagas disease.

Results

Mapping of kDNA Integration Sites in the Human Genome

Upon entry of *T. cruzi* into the body, the infective trypomastigotes are taken up by mononuclear phagocytes.

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The intruding flagellate may be destroyed in the phagocytes, but many of the internalized parasites replicate as amastigotes before returning to the trypomastigote forms that then burst out of the host cell to invade any tissue or cell type. A vast majority of the parasite's existence within a vertebrate is spent hidden from the host immune system inside muscle cells. Indeed, *T. cruzi* can persist for decades in this setting without causing the host significant damage. We observed that treatment of chronically infected rabbits with a trypanocidal drug curtailed tissue parasitism, but it did not stop the progressively destructive myocarditis and peripheral nerve system ganglionitis, hallmarks of Chagas disease (Teixeira et al., 1990). What could be sustaining the active destruction of the heart cells? In order to answer this question, we postulated that some rate of genetic transfer could occur from the parasite to the host genome and that resulting mutation could explain the persistence of autoimmune-driven lesions (Leon and Engman, 2003). Therefore, we decided to search for parasite DNA in the genome of Chagas patients.

We analyzed genomic DNA samples from the blood of 13 Chagas heart disease patients that had shown antibody specific to *T. cruzi* antigens. These patients had vector-transmitted *T. cruzi* infections for over 30 years and had recently manifested the clinical symptoms of chronic Chagas heart disease (Lauria-Pires et al., 2000). These entire samples harbored parasite DNA as judged by PCR amplification with primers for both *T. cruzi* nuclear DNA (nDNA) and kDNA minicircle (Figure 1A). Southern hybridizations were performed on genomic DNA from these Chagas patients. In a typical example, the DNA sample from Chagas patient 245 revealed 400 and 100 bp bands with the kDNA minicircle probe only, whereas digested and catenated minicircles revealed high molecular weight bands of >4 kb and 1.4 kb, respectively (Figure 1B). In order to determine the precise site of kDNA integration, genomic DNA templates from the 13 individual Chagas patients were subjected to 5′ RACE. Analysis of the resulting products revealed fragments of kDNA minicircles of variable length integrated into the genome of each of the 13 patients at one or more loci (Table 1). Inspection of the integration sites showed that insertions were flanked by a CCAACAC motif in ten out of 18 clones obtained. The kDNA was integrated at the β-globin locus on chromosome 11 in nine out of 13 of the Chagas patients. Complete open reading frames were deduced from these sequences, with translation of putative chimeric proteins.

In order to confirm and further dissect the process of genetic transfer from *T. cruzi* to Chagas patients, we moved our studies into rabbits, which are amenable to infection by *T. cruzi*, and chickens, which are refractory to persistent infection.

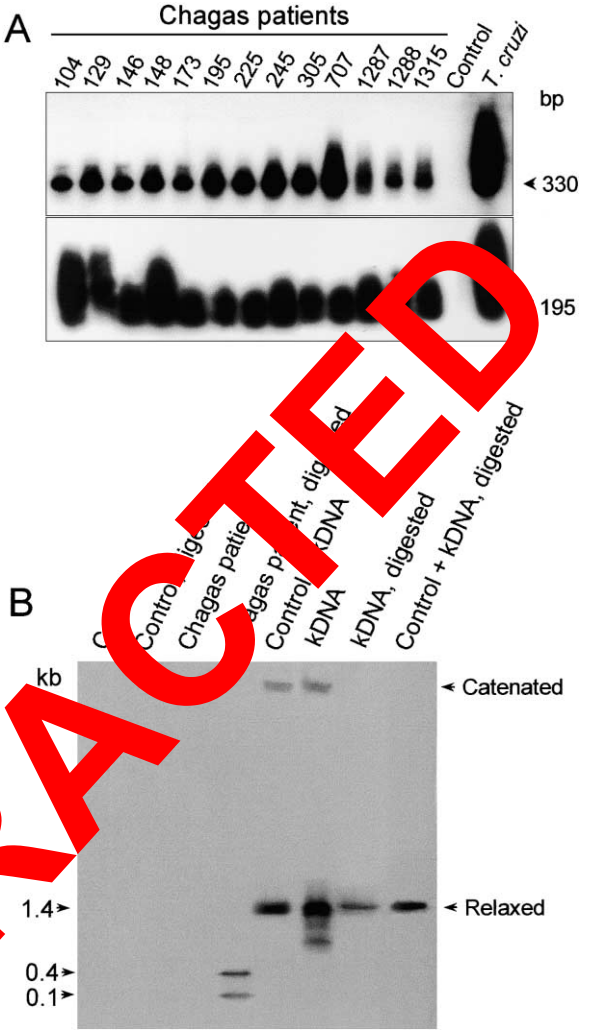

Figure 1. Genetic Markers of *T. cruzi* Infections and Genomic kDNA Integration in Chronic Chagas Patients

(A) Evidence of the persisting *T. cruzi* infections. PCR amplification products obtained from template DNA of 13 Chagas heart disease patients and specific sets of kDNA (S35/S36; upper panel) and nDNA (Tcz1/Tcz2; lower panel) primers, which hybridized with specific internal probes S67 and Tcz3, respectively, on blots of 1% agarose gels. The size markers indicate the minimal unit size of amplification in each case.

(B) Southern hybridization of integrated kDNA revealed by a minicircle (kCR) probe. A 0.7% agarose gel was used to analyze undigested DNA or EcoRI and BamHI double-digested DNA from the blood of Chagas disease patient 245. After size separation through a 0.7% agarose gel, the DNA was stained with ethidium bromide, blotted, and probed with the kCR oligonucleotide. DNA from a noninfected human donor and purified kDNA were included as controls. The predicted sizes of catenated and relaxed or linear minicircles are indicated.

Integration of *T. cruzi* kDNA Minicircle Sequences into the Rabbit Genome

The kDNA integration sites associated with modifications in the human genome could represent a critical biological element in host-pathogen interactions leading to the chronic clinical manifestations of Chagas disease. Initially we sought to reproduce the phenomenon in the rabbit model infection system (Teixeira, 1986).

The active process of genetic transfer between *T. cruzi* and rabbit was initiated with the examination of DNA from tissues extracted from eight animals that had been infected for six months to three years. Digests of DNA extracted from blood, heart, skeletal muscle, liver, intestine, and kidney were hybridized with the 122 bp constant region (kCR) probe of the *T. cruzi* kDNA minicircle (Figure 2A). A 2.2 kb band was obtained in the heart-

Table 1. Mapping of the Sites of *Trypanosoma cruzi* kDNA Integration in the Chromosomes of Mammals and Birds

Samples[a]	GenBank	Sequence Size/kDNA	Integration Sequence	Chromosome	Locus	ORF's Translated Chimeric Protein[b]
Chagas Disease Patients						
104 c2	AY485269	490/1–360	CCAAC-ACTGC	16	16p13.3	NF
129 c1	AY490889	174/131–174	GTTGGC-ATCT	11	β-globin	NF
129-c4	AY490892	546/1–532	GTTGGTGGC[illegible]G	[illegible]	Unknown	*MHQPQSNHQPRSNXQPQXNHQLQSNXQPQSNXTCHQPQSNHQFQSNHQPQSNHQLQSNHQPQSNHQPQS NHQPQSNHTSRKRTPKMTI*
146-c19	AY490890	580/99–580	GGAACC-ACCACCA	[illegible]	[illegible]obin	*MVRLGVVVRLKLVVRLGLVVRLKLVVRLGLVVRLGLVHLTRALYGHFRGAFTGGVVRLGLVVRLKLVVRLGLVVR LGLVVRLGLVHLTRALYGHFRGAFTGGVVRLKLVVRLGLVVRLGLGGF*DLGLVGSELKFGGSXWGLVGSD
146-c91	AY490895	313/1–224	GGTGGTCC-AAAC	1	β-globin	NF
148-c26	AY490891	231/1–100	AGATGCC-AACAC	16	[illegible]p13.3	*MYRVRCPCIMYRVRCPCIMY*GGDANTADETVKRLSQLQWDEASLMKEARVVVDKPRCTNHLLSQREA
173-c5	AY490893	96/1–79	ATTCCGAA-CCCA	8	8 q23	[illegible]
173-c33	AY490897	156/1–76	GAATTTTA-CAGA	11	β-globin	[illegible]
173-c56	AY490894	169/94–169	GTTAAC-AAATTG	X	Xp21.1	*MHE[illegible]SAKKLNAP*PKTKFPQFLNLRIHLKLYPPLPQYNNSI
173-c68	AY490896	192/1–73	TCCGAA-TTCTG	11	β-gl[illegible]	*MHEII[illegible]NAPPKS*IISEFCSTRLLPNLNHKPTNH
195-c7	AY490898	173/1–130	ATTATAC-AAACG	11	β-[illegible]in	NF
225-c12	AY490899	224/1–141	TTCCGAA-TTTTAC	X	Xp21.1	[illegible]
245-c2	AY490900	593/1–461	CACA-CCTTCCG	17	17q21-q22	[illegible]*SNLPQSRRTELVAXPTPIEPPISIEPPTPIKPPTPMEPTSATPTPIEPHQPQSNHTFRLVTRGH WSF[illegible]WSATSRAGLLGIRGXEVAIWRLHAW*
305-c2	AY490906	286/177–286	GGAAAG-GGTTCC	11	β-globin	*MHQP[illegible]NHQLQSNHQ[illegible]SNHQLQSNHQPQSNPFLDYLLRIQPEKGSERWPG VQA[illegible]THFEDPQ[illegible]FGLPSPGCCDHLESEPEDGR*
707-c12	AY490905	280/154–280	ATTCGAT-TGGTT	11	β-globin	NF
1287-c42	AY490901	311/1–180	GAGATGC-ACAAC	11	β-globin	NF
1288-c18	AY490902	315/1–138	GAA-CCCCTTTC	11	β-globin	NF
1315-c1	AY490904	523/1–395	GAGATGC-ACAAC	11	β-globin	NF
Chagas Disease Rabbits						
OCE9-c41	AY488498	223/132–223	GTGTTG-GCATCT	NF	Kinesin	*MPMYNVRVRCPCIMYGGD[illegible]*NTADETVKRLSQ[illegible]DEASLMKEAGWLWTSLGVPITSFHKERP
OCE10-c28	AY488499	254/1–123	ACGGG-GGAAG	1	β-globin	*MYGGDTPTPIEPPTPIEPPT[illegible]PPSPIEPPTSIEPP[illegible]EPHP*
OCE10-c31	AY488500	356/1–256	AGATGC-CAACA	NF	Kinesin	*MPMYNVRVRCPCIMYGGDAN[illegible]VKRLSQLQ[illegible]LMKEAGWLWTSLGVPITSFHKERP*
OCE12-c22	AY488501	577/1–564	GGTGGG-CAGGGC	1	β-globin	*MHQPQSNHQFQSNHQPQSNHQFQSNHQPQS[illegible]QLQS[illegible]HQPQSNHQLQ*
OCE12-c31	AY488502	177/1–46	GATGC-CAACAC	1	β-globin	*MYGGDANTADETVKRLSQLQWDEASLMKE[illegible]WLWTSLGV[illegible]SFHKERP*
OCE12-c18	AY488503	422/1–321	GNTGG-NGGGAA	NF	NF	*MEPXWVTPXPXEPNVQSXPAIQAGARSR[illegible]TTPIEPNL[illegible]NHSQSPPMAISSYTNPNRTQATPLRTDG TPIGTHSVHQPQSEPIPHTNPNRT*
OCAV-c13[c]	AF400668	370/1–220	CAACC-GAAGTT	1	NF	
OCAV-c12[c]	AF399841	688/237–688	—	1	NF	MQDAGASGSGSARDATP*TPIEPLTPIEPPTPRTTN[illegible]RTTNPT[illegible]TNPNP[illegible]PNRTTNPNRTTNFNRTTNPN RTTNSNRTTNPNRTTNPNQNHQPQSNHQPQSNH[illegible]QSNHQPQSN[illegible]*
OCAV-c14[c]	AF415293	1066	TGCCAC-ACCAAC	1	β-globin	NF
Chicks Hatched from *Trypanosoma cruzi*-Infected Eggs						
GG2-c43	AY237305	492/1–112	ACCACA-ATTTCT	NF	NF	NF
GG3-c10	AY237306	573/1–87	AAGC-CCTATA	NF	NF	NF
GG4-c8	AY289115	311/1–31	TATAGG-GGCAGT	NF	NF	NF

[a] Clones derived from Chagas disease patients, from adult *T. cruzi*-infected rabbit, or from offspring of *T. cruzi*-infected does yielded sequences showing kDNA integrated in loci enmeshed in LINE-1 elements dispersed in specific host chromosomes. Clones OCAV-c12-14 represent the 5′ end of a rabbit SINE complete repeat unit and LINE repeat unit, showing integrated kDNA (GenBank accession number AC144399).

[b] Translated aa sequence from integrated kDNA is shown in *italics*, and its transition to host DNA is underlined.

[c] Consensus sequence depicted in model Figure 2B. NF = integration not found in coding regions, although it can cause gene disruption by integration in noncoding regions.

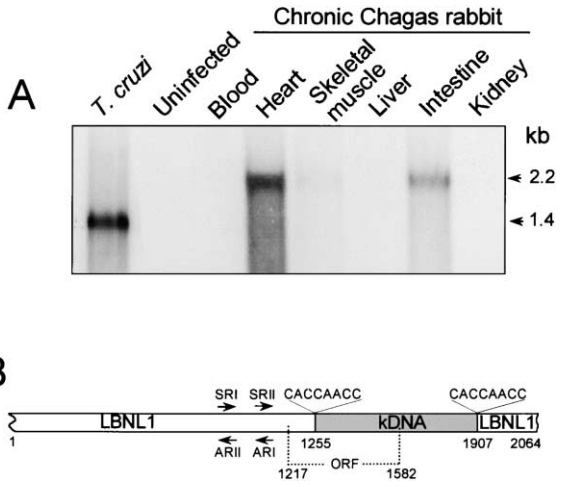

Figure 2. Integration of kDNA Minicircle Sequence into the Genome of a Chagas Disease Rabbit

(A) Hybridization of rabbit DNA with a specific kDNA probe. EcoRI digested DNA (20 μg) separated in 0.7% agarose gel was used for Southern hybridization with 1 μg of *T. cruzi* kinetoplast kCR probe. (B) Schematic representation of kDNA integration into rabbit DNA. Arrows show primers used in 5′ RACE to detect kDNA insertion into the rabbit genomic clone LBNL1. Integration of CCA/ACC-rich kDNA (bp 1255–1907) occurred within rabbit DNA showing attachment sites of direct short CACCAACC repeats. An ORF spans the chimeric sequence, bp 1217–1582.

and large intestine-derived DNAs, distinct from the m[illegible]-circle unit-sized 1.4 kb band hybridizing in parasit[illegible]NA alone. DNA extracted from tissues of an uninfe[illegible]ted

bit showed no hybridization with the kCR probe. The tissue samples showed no hybridization with the other *T. cruzi*-specific nuclear DNA probes for high-copy number genes or repetitive sequences (Moser et al., 1989; Requena et al., 1992; Murthy et al., 1992). To characterize the 2.2 kb band, 5′ RACE was employed on DNA from infected rabbit heart. This approach generated sequence containing both arms of rabbit DNA flanking the kDNA insertion (Figure 2B). Integration of a CCA/ACC-rich kDNA fragment (bp 1255–1907) oc[illegible]attachment sites of direct CACCAACC repe[illegible]within t[illegible]abbit DNA. The extreme 3′ region (1908–2[illegible]bp) has l[illegible]mology with the LBNL1-125D4 clo[illegible](Gen[illegible]k ac[illegible]ssion number AC144399), a rabb[illegible]LINE con[illegible]g inter-spersed SINE repeats (Pric[illegible]t al.,[illegible]92). An ORF initiating in the host DNA and e[illegible]g bev[illegible]d the kDNA integration site (1217-[illegible]2 bp)[illegible]slat[illegible]a putative chimeric r45-like anti[illegible](GenBan[illegible]cession number AAR24603.1).

The de novo transfer o[illegible]NA to the rabbit genome supports th[illegible]tention tha[illegible]genetic transfer may be part and p[illegible]cel of the normal course of a *T. cruzi* vertebrate infe[illegible]on. To ful[illegible]r emphasize this point, we took advantage[illegible]our ab[illegible]y to infect rabbit and chicken embryos wit[illegible].

T. [illegible]rowth in Embryonic Stem Cells

The [illegible]asion of embryonic stem cells by *T. cruzi* has not be[illegible]demonstrated previously. We first explored the [illegible]ossibility of *T. cruzi* uptake by stem cells in vitro.

[illegible]abbit embryo stem cells were readily invaded in vitro

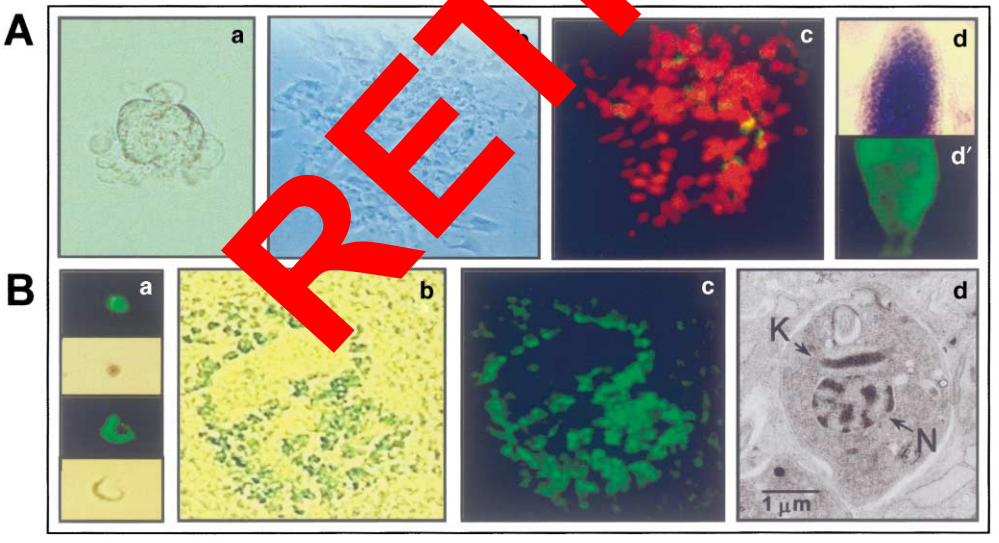

Figure 3. Growth of β-Galactosidase Expressing *T. cruzi* in Embryonic Stem Cells of Mammal and Birds

(A) *T. cruzi* growth in rabbit embryonic stem cells. (Aa) A rabbit zygote at stage 2 days postcoitus blasted off stem cells that adhered to the surface and was exposed to 1,000 trypomastigotes. (Ab) A multilayer of embryonic stem cells formed on the fourth day in culture (phase contrast, magnification 200×). (Ac) Same field, in which nuclei of stem cells are counterstained with ethidium bromide, showing immunofluorescence-stained (arrow) intracellular amastigotes. (Ad) Embryonic stem cell showing amastigotes stained blue and counterstained with eosin; (Ad′) Same cell, showing immunofluorescent intracellular amastigotes of *T. cruzi* recognized by a specific antibody (magnification, 800×).
(B) *T. cruzi* growth in endodermal and mesodermal cells of a 4-day-old chicken embryo. (Ba) Round amastigote and slender trypomastigotes forms are stained fluorescent green as a result of recognition by a specific antibody from a patient with Chagas disease, and brown forms are the same unstained *T. cruzi* stages seen by phase contrast microscopy (magnification, 1200×). (Bb) Paraffin-embedded section showing blue X-Gal stained cells in the endoderm and mesoderm tissues of a 4-day-old embryo (magnification, 200×). (Bc) Immunofluorescence-stained *T. cruzi*-infected cells colocalized in the same embryo tissue section. (Bd) Electron micrograph showing an intracelullar amastigote. k, kinetoplast. n, nucleus.

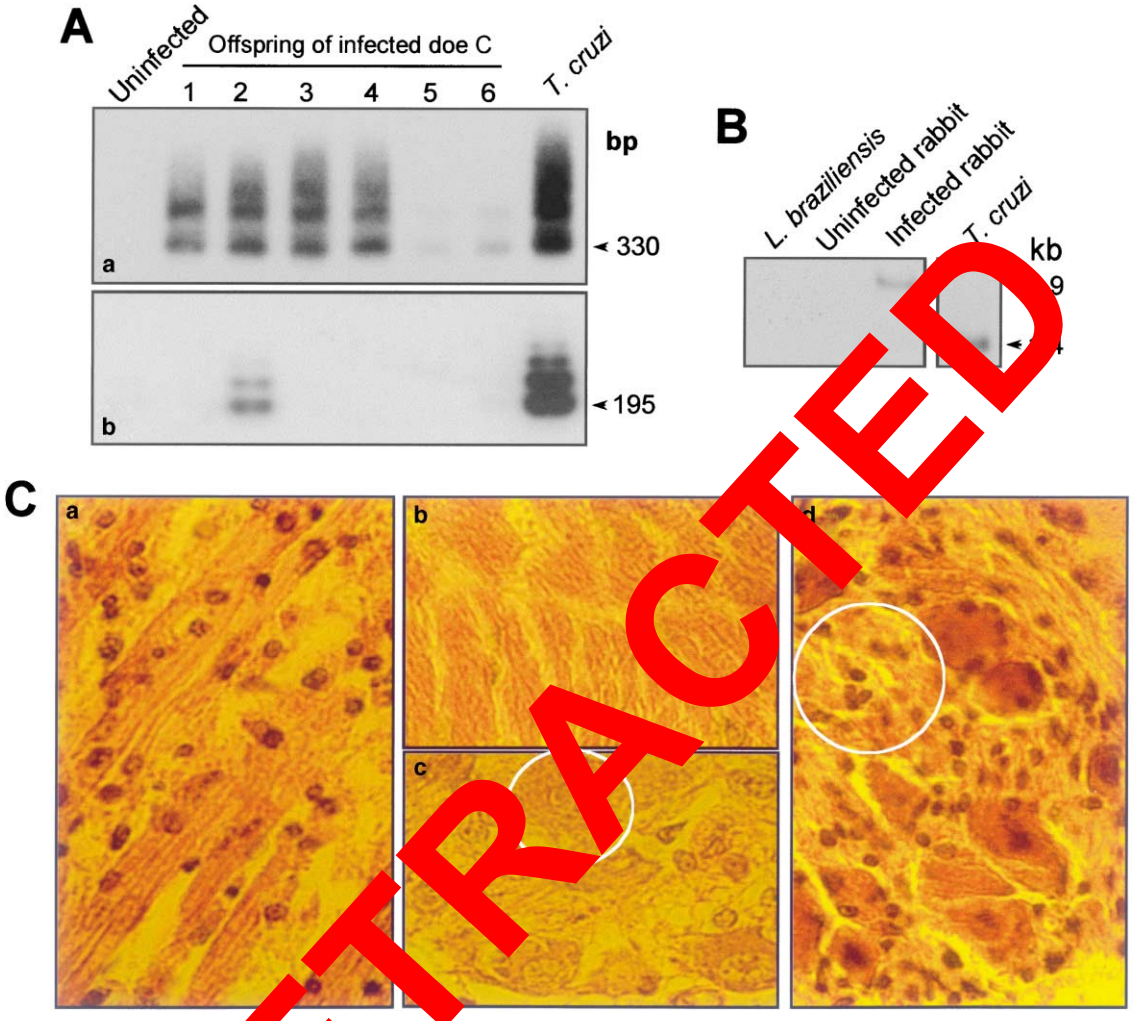

Figure 4. Genetic Marke[r] of *T. cruzi* Infection in Offspring of Chagas Disease Rabbits, kDNA Integration, and Pathology

(A) Evidence of the persist[ence] of *T. cruzi* in[fect]ions. Specific hybridization of PCR amplification products from template DNA obtained from offspring of Chagas[ic] d[oe] using sets of *T. cruzi* nDNA and kDNA specific primers. DNA products were resolved in 1% agarose gels. (Aa) PCR analysis of kD[NA] amplifi[cation] shows bands of 330 bp and its catamers from the parasite DNA and from genomic DNA of six progeny with hybridizat[ion] with t[he] kCR probe. (Ab) PCR analysis of nDNA amplification shows bands of 195 bp and its catamers formed with parasite DNA and fro[m] nom[ic] [of] spring 2 after hybridization with the specific internal probe.

(B) Southern hy[bri]dization showing integration of kDNA minicircles into the genome of offspring C1 from a *T. cruzi*-infected doe. Test and control DNA (20 μg) [were] digested with EcoRI along with 10 ng of *T. cruzi* or of *L. braziliensis* DNA for Southern hybridization against the kCR probe. Separation of [the] products was achieved as described in Figure 2A.

(C) Destructive myocarditis and ganglionitis in two week-old offspring 1 from doe C. (Ca) Histopathological section showing mononuclear cell infiltration and lyses of target heart cells. Note the round lymphocytes adhered to the surface of the target cells. (Cb and Cc) Normal histological features of myocardium and intracardiac ganglion cells from a control offspring of a noninfected rabbit. (Cd) Intracardiac parasympathetic ganglion showing mononuclear cell infiltration and neuron drop out (circle).

by β-galactosidase expressing *T. cruzi* trypomastigotes. Rabbit zygote blast cells actively engulfed the protozoa, with penetration of the host plasma membrane 6 hr after adhering to the plastic surface (Figure 3Aa). After the fourth day in culture, dividing amastigotes were identified in the growing embryo stem cells (Figures 3Ab and 3Ac). Similar host cell and *T. cruzi* growth kinetics were observed with infected chicken embryonic cells. On the eighth day in culture, at 37°C, the stem cells full of amastigotes were easily identified by immunofluorescence and X-Gal staining (Figures 3Ad and 3Ad′). Using this immunohistochemical approach, we confirmed amastigotes of *T. cruzi* in chicken embryo endoderm and mesoderm tissues (Figures 3Bb and 3Bc) at stages corresponding to 4 and 8 days old, which had been infected prior to incubation. The blue-stained cells in the tissues that were examined under the electron microscope showed typical intracellular *T. cruzi* forms (Figure 3Bd).

The permissiveness of the embryonic stem cells to *T. cruzi* infection at the blastula stage (2-day-old zygote) is an indication that differentiating germline cells in the genital crest, which appear at days 4–8.5 of gestation (Ginsburg, 1997; Kagami et al., 1997), could contain kDNA minicircles due to invasion. Thus, these cells are candidates for horizontal or germline transfer of parasite DNA.

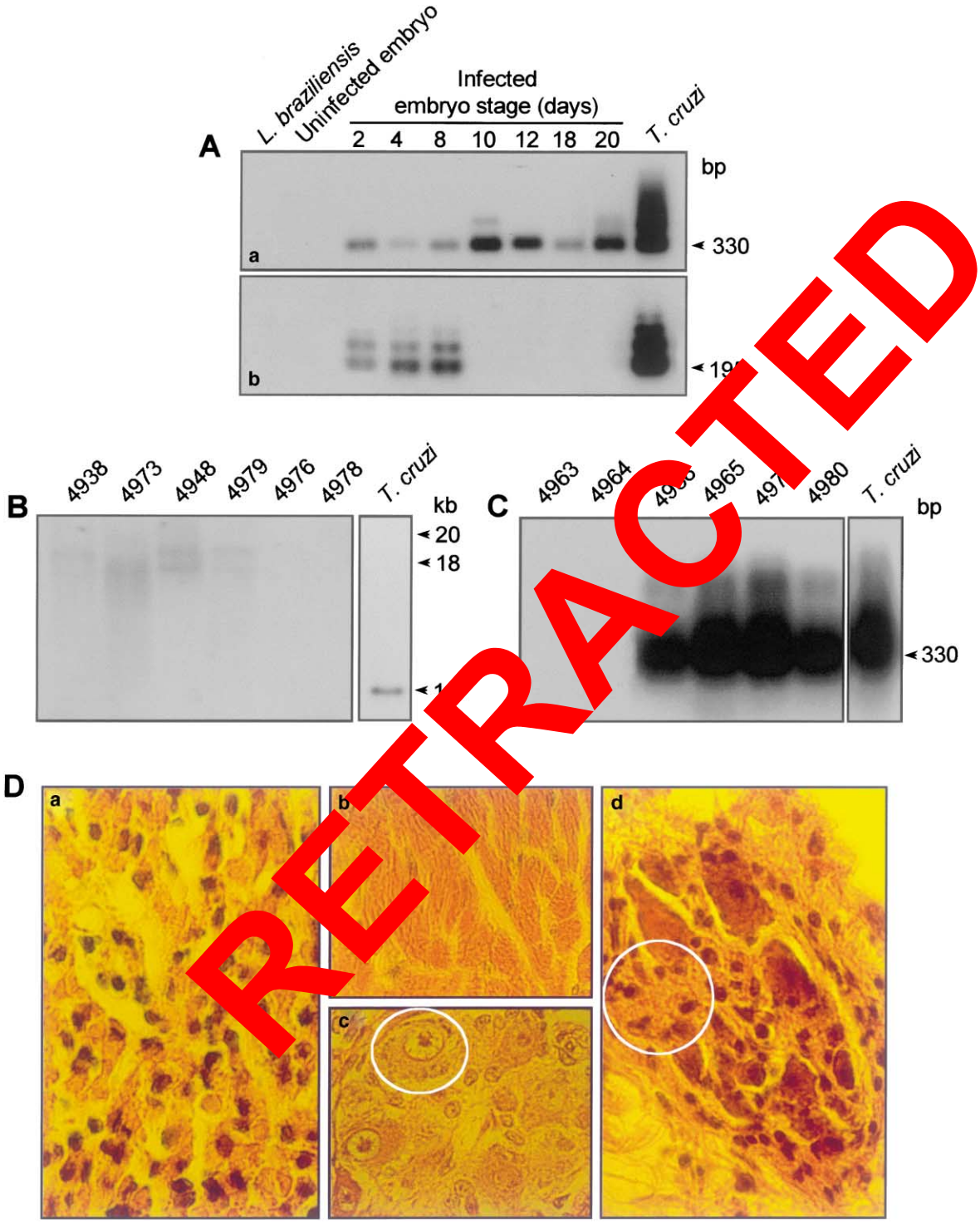

Figure 5. Evidence of kDNA Integration in Germline Cells and Tissues from Birds Hatched from *T. cruzi*-Infected Eggs, with Accompanying Pathology

(A) Establishment of *T. cruzi* infection early in embryonic development followed by loss at 10 days of incubation. PCR, size separation, blotting, and hybridization were performed as described in Figure 1A. (Aa) Hybridization of PCR amplified bands of kDNA minicircles. kDNA products were amplified from DNAs harvested from tissues at several stages in the embryonic development of the chicken as indicated. (Ab) Hybridization of PCR amplification of sequences of nDNA. Bands of 195 bp were diagnostic of nDNA presence in the template DNA.

(B) Southern hybridization of EcoRI digests of genomic DNA derived from *T. cruzi*-infected or uninfected eggs with a kDNA specific probe. Band sizes of approximately 20 and 18 kb formed with DNA from *T. cruzi*-infected birds 4938, 4973, 4948, and 4079 but not with DNA from uninfected, control birds 4976 and 4978. The positive control consisted of 10 ng of *T. cruzi* DNA.

(C) Integration of kDNA fragments into the avian germline. PCR hybridization analysis of template DNAs from sperm (4938 and 4965) and from

Persistence of kDNA of *T. cruzi* in Tissues of Newborn Rabbits

In addition to insect-vector transmission, *T. cruzi* infections can be acquired transplacentally in human Chagas disease (Schenone et al., 2001). By this mode of transmission, the blood forms of the parasite transverse the placental barrier and through the umbilical cord to reach the fetus, which then shows tissue parasitism (Teixeira, 1987). This knowledge prompted us to examine congenital infection and subsequent kDNA integration in rabbit litters from chronically infected Chagas rabbits. Previous in vitro demonstration of the *T. cruzi* infection of 2-day-old rabbit zygote embryo cells indicated to us that the in utero embryonic infection could lead to kDNA integration in primordial germlines.

Four sexually mature does and two bucks were infected with *T. cruzi* trypomastigotes and crossbred during chronic infection. After three pregnancies, the does with chronic *T. cruzi* infections delivered 104 litters (26 ± six per doe), among which 23 (22.1%) were stillborn. Three control, uninfected does in three pregnancies delivered 96 litters (32 ± 3 per doe), among which 22 (22.9%) were stillborn. PCR was carried out on DNA either from specific tissues of stillborn animals or from blood cells of individual surviving offspring of the *T. cruzi*-infected rabbits for both *T. cruzi* nDNA and kDNA (Figure 4A and data not shown). A sample showing the presence of kDNA but not nDNA (Figure 4A, offspring 1 of doe C) was examined by Southern hybridization of EcoRI-digested genomic DNA with a kDNA probe (Figure 4B). A band that was larger in size than minicircle was detected, indicative of integration event. No bands were detected with nDNA or minicircle probes. The control, uninfected rabbit DNA showed the absence of bands that hybridized with nDNA or kDNA probes. Out of the 104 surviving offspring from chronically infected parents, 15 (14.4%) contained nDNA, and 24 (23%) contained kDNA by the PCR assay. Nine stillborn offspring yielded DNA from heart, skeletal muscle, liver, spleen, and large and small intestine, and each tissue type found specific band by hybridizing amplification products with kCR probe (data not shown). Genomic DNAs of kDNA-positive offspring were subjected to 5′ RACE, yielding six integration sites of the kDNA fragments (Table 1). In three out of six cases, the mutated kDNA entered the β-globin cluster in chromosome 1, whereas offspring OCE12 yielded two clones showing kDNA in different frames of the β-globin locus. Three of these clones showed the CCAACA motif flanking integration sites and potential ORFs for chimeric proteins. To determine whether the living infection was needed for kDNA integration into the host genome, we inoculated purified or cloned minicircle sequences intravenously into rabbits and monitored animals' DNA weekly for 3 months. kDNA products were amplified from blood DNA up to but not beyond the third week postinoculation from these rabbits. Tissue-specific histopathological lesions in muscle tissues, which were usually extensive in the peripheral nervous systems of stillborn offspring of *T. cruzi*-infected rabbits, were seen. Myocarditis and ganglionitis, which are typical of Chagas disease in humans and in rabbits (Teixeira et al., 1983), were seen in offspring 1 of doe C, consisting of mononuclear cell infiltration and lyses of parasite-free target host cells (Figure 4C). None of these lesions was present in the spleen, in the liver, or in the kidney of offspring from Chagas disease rabbits or in any tissue of control offspring from uninfected rabbits (data not shown).

This set of experiments demonstrates the high frequency of horizontal kDNA transfer in vivo into the vertebrate host genome. The resulting offspring harbor persistent living infection (Teixeira, 1987), but the presence of kDNA fragments in different tissue types suggests that some integrations occurred shortly after parasite invasion, resulting in the possibility of integrated kDNA throughout the germline of the host.

kDNA Integration into the Germline of Chickens

To clearly dissociate the kDNA integration event from the presence of active infection, we continued our *T. cruzi* invasion studies in the chicken system. This approach was explored to dissociate the kDNA integration from cryptic *T. cruzi* infection. We demonstrated previously that these vertebrates are susceptible to *T. cruzi* only at the early embryonic stage, after which they are refractory to persistent infection. Therefore, a kDNA integration event occurring early in the embryonic developmental process could result in the generation of a mature chicken with kDNA integrated into gonadal tissue.

Thirty-six fertile chicken eggs were each injected with 100 *T. cruzi* trypomastigotes. The embryo tissue collected on the second, fourth, and eighth days postinfection yielded nDNA and kDNA amplifications; however, tissue collected on the tenth, 12th, 18th, and 20th days of incubation yielded amplification products only for kDNA (Figure 5A), indicative of the clearance of active infection. Two roosters (4938 and 4979) and two hens (4973 and 4948) that hatched from *T. cruzi*-infected eggs showed positive hybridization bands with the kCR probe in a Southern blot performed on DNA isolated from sperm and from nonfertilized eggs (Figure 5B), with their pattern of migration differing from that of free-relaxed or of free-catenated minicircle DNA. These birds were raised for crossbreeding. In the control group, 14 fertile chicken eggs were subjected to PCR, and neither nDNA nor kDNA was detected (data not shown). Additionally, we inoculated naked minicircle or cloned minicircle sequences in the air chamber of 30 fertile chicken eggs. Absence of PCR amplification products from these em-

nonfertilized eggs (4970 and 4980) from experimentally infected birds, compared to control birds 4963 and 4964. Probe and controls used are described in (B).

(D) Destructive myocarditis and ganglionitis in 2-week-old F1 chick 1072. (Da) Histopathological section showing mononuclear cell infiltration and lyses of target heart cells, similar to that described in Figure 4C. (Db and Dc) Normal histological features of myocardium and intracardiac ganglion cells from a control offspring of a noninfected chick. (Dd) Section of an intracardiac parasympathetic ganglion showing lymphocyte infiltration and drop out of neuronal cells (circle).

bryos' template DNAs tested weekly prior to hatching indicated that, as in rabbits, transfer of minicircle kDNA sequences to a bird's genome requires a living *T. cruzi* infection (data not shown). In order to determine whether kDNA-transfected birds harbored the *T. cruzi*-specific DNA in the germline cells, we collected sperm from 4938 and 4965, and eggs at an early stage of development from the ovary of 4970 and 4980. DNA templates that were extracted from these samples amplified kDNA but not nDNA (Figure 5C). DNA from testes and ovaries of control, uninfected birds 4963 and 4964 did not yield products. Histopathological lesions in muscle tissues and in peripheral nervous systems were seen in offspring that hatched from *T. cruzi*-infected eggs. Myocarditis and ganglionitis were visible (Figure 5D), similar to those lesions described in a rabbit's offspring. These diagnostic lesions were absent in tissues of control offspring from chicks hatched from uninfected eggs (data not shown). This rejection of parasite-free target host cells has been observed in adult birds with kDNA integration (C.G., N.N., A.C.R., M.R.S.-A., F.M., and A.R.L.T., unpublished data).

These experiments document the generation and confirmation of chickens with kDNA integrated into their germline cells in the absence of persistent infection. The presence of kDNA with mobility differential from native minicircles in the gonadal DNA of individual chickens combined with the absence of *T. cruzi* nDNA attests to the success of the integration event and of the subsequent eradication of *T. cruzi*.

Germline Transmission of Integrated kDNA in *Gallus gallus*

With the creation of hens and roosters with integrated kDNA in their ova and sperm, we were poised to determine the transmission of integrated kDNA to the resulting progeny independent of persistent, cryptic *T. cruzi* infection.

The kDNA transfected rooster 4938 and hen 4973 and 4948 were bred to produce vertically germline transmission of kDNA to their offspring. Two chicks that were born from these crosses carried the kDNA in their genomes, as shown by amplification from blood cell DNA (data not shown). kDNA-positive F1 birds were crossed to obtain F2 hybrids. Pedigrees of kDNA-transfected progeny are shown in Figure 6A. kDNA transfected rooster 4979, and control, uninfected hens 4976 and 4978 were bred to detect the frequency of vertical inheritance of kDNA to F1 and F2 offspring from a single kDNA donor parent (Figure 6B). kDNA was detected in the genomes of all chicks that were born from these crosses, indicating that integrated kDNA can be inherited through the male (Figure 6Ca). In a control PCR for nDNA, no bands were obtained from any of the offspring DNAs (Figure 6Cb). Cloning and sequencing revealed kDNA integrated into the host DNA of hens 4948 and 4973 and offspring 1071 (Table 1).

The separation of the kDNA integration event from active infection with *T. cruzi* quells any doubt that our observations are spurious. The fertility of roosters and hens harboring specific integration events bodes well for additional studies on the biological effects of *T. cruzi* DNA acquisition.

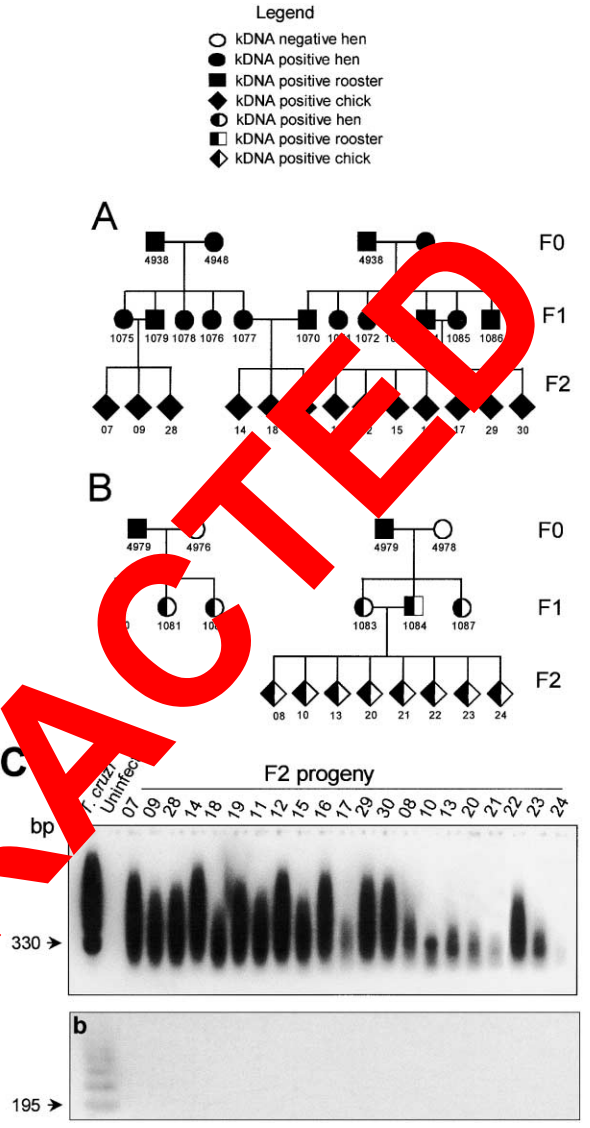

Figure 6. Pedigree of Chickens Carrying Integrated kDNA

(A) Parentals kDNA-positive rooster 4938 kDNA-positive hen 4948 (F0) were crossed, yielding five offspring each carrying the kDNA genotype. Similarly, kDNA-positive rooster 4938 was crossed with kDNA-positive hen 4973, yielding seven offspring (F1) each carrying the kDNA genotype. Further crossings of F1 resulted in offspring, each carrying homozygous kDNA genotype (F2).

(B) Parentals kDNA-positive rooster 4979 was crossed with kDNA-negative hen 4976 (F0), yielding three offspring each carrying the kDNA genotype. Similarly, kDNA positive rooster 4979 was crossed with kDNA negative hen 4978, yielding three offspring carrying the kDNA genotype (F1). Further crossings of F1 positive rooster with negative hen resulted in offspring carrying hemizygous kDNA genotype.

(C) Evidence of persisting kDNA but not nDNA in tissues from progeny of birds that had hatched from *T. cruzi* infected eggs. The PCR hybridization assay was performed as described in Figure 1A. (Ca) Hybridization of PCR amplification of kDNA minicircles utilizing the kDNA primer set showed band size 330 bp and its catamers in each F2 progeny, which were not formed in uninfected, control DNA. Controls consisted of 10 ng of *T. cruzi* DNA and 20 μg of uninfected chick DNA. (Cb) Absence of *T. cruzi* nDNA in progeny of germline kDNA parents. The 195 nt band and its catamers formed with the *T. cruzi* DNA only.

Discussion

Here we report integration sites of *T. cruzi* DNA into vertebrate host genomes as a consequence of infection. Fragments of the parasite's specialized mitochondrial minicircle DNA were found integrated frequently within LINE-1 elements or interrupting β-globin genes. We document integrations that have occurred during natural infection in humans and have reproduced experimentally the analogous kDNA transfer events in rabbits and chicken. With the establishment of germline kDNA integration in chickens, we have demonstrated vertical transfer of *T. cruzi* DNA to infection-free progeny. These integration events may be a key to understanding aspects of chronic Chagas disease manifestation, which can take decades to present in humans. We present a model for *T. cruzi* infection, including flagrant genetic transfer between eukaryotes that is readily detected in Chagas patients and in experimentally infected rabbits and chickens.

From a historical perspective, the transfer of DNA between organisms of distant evolutionary relationship is similar to the events postulated during the formation of eukaryotic cells as we now know them. The specific detection of kDNA minicircle fragments integrated into the genomes of vertebrate hosts is reminiscent of the process of bacterial assimilation of organelles such as mitochondria and chloroplasts (Margulis and Saga 2002; Hannaert et al., 2003). Our detection of minicircle DNA versus parasite DNA of nuclear origin does not eliminate the possibility of true horizontal gene transfer between the parasite and host, in which transferred gene is expressed in its new cellular environment. The sheer number of minicircles, each with four conserved regions containing CA-rich sequence motifs, may be the dominant characteristic influencing the high frequency of this serendipitous event. In the case of minicircle integration, the guide RNA genes are not likely to be expressed in their new location, but rather, the integrated fragments act as disadvantageously by their disruption of endogenous genes.

Horizontal DNA transfer is a natural consequence of infection in the vertebrate host. The intracellular invasion by *T. cruzi* is required for minicircles to integrate into the vertebrate host cell genome. Integration does not occur in the invertebrate vector genome, which does not include an intracellular stage (our unpublished data). Therefore, intracellular growth and differentiation is included among the environmental factors associated with kDNA transfer, integration, and continuity in progeny of the host species. This is a highly specific phenomenon, dependent on four "shuttle vectors": (1) the triatomine shuttle that transmits the infection to mammals, (2) the intracellular protozoan carrying kDNA to the host genome, (3) the presence of CA-rich motifs in the target host's genome and parasite's mitochondrial DNA mediating homologous recombinantion as a mechanism of kDNA integration, and (4) host germline cells that pass exogenous DNA to the progeny in the absence of continued parasitemia.

The integration of kDNA fragments into LINE-1 elements has implications for the further mobilization of foreign DNA within the host genome. Vertebrate genomes contain repetitive long and short elements (LINEs and SINEs) that persist by vertical transmission within a host. The human genome includes 535 LINEs belonging to the Ta subset and 415 belonging to the pre-Ta subset. Thirty-nine such elements of the Ta subset and 22 of the pre-Ta are full-length elements with intact ORFs that are candidate-active LINEs (Feng et al., 1996). These elements are likely progenitors of mutagenic insertions into β-globin and other genes and provide a means for the mobilization of DNA sequences around the genome and for exon shuffling (International Human Genome Sequencing Consortium, 2001). Upstream promoters initiate L1 transcription generally confined to germline (Trelogan and Martin, 1995), but a somatic L1 retrotransposition event has been correlated with disease (Kazazian and Moran, 1998). The high specificity of kDNA integration with genome of humans and rabbits was observed in 20 of 20 samples tested. We believe that kDNA insertion mutagenesis leading to modification of the host cell phenotype may correlate with autoimmune-driven lesions (Ljunggren and Engman, 2003), or they may be carried out by kDNA mutations associated with retrotransposition of LINE-1 elements (A. Simões-Barbosa, F.R. Argañaraz, Ana Maria Barros, A.C.R., Marian R. Souza-Atz, N.N., R.J.N., and A.R.L.T., unpublished data). Disease manifestations (Miki, 1998).

Congenital transmission of the *T. cruzi* infection to offspring from mothers with acute or chronic disease can be documented in subsequent generations, but transfer and integration of kDNA in the progeny has never been explored. Acute infections in infants and children usually go unrecognized and subside spontaneously. The *T. cruzi*-infected child usually does not become sick at a time when tissue parasitism is fairly high but may develop striking chagasic lesions several decades later when the parasite is difficult to find (Teixeira et al., 1978). The pathogenesis of some Chagas disease lesions has been associated with mononuclear cell infiltrates and rejection of amastigote-free target host cells (Teixeira et al., 1996). Certainly, disease manifestation may not be dependent exclusively on the parasite's direct action upon a host cell, and autoimmune rejection is likely to play a role in the pathogenesis of Chagas disease (Girones and Fresno, 2001). The role of autoimmunity in Chagas disease will be examined in ongoing experiments aimed at the detection of parasite-independent heart homograft rejection in congenic kDNA-mutated chicken strains.

The result of disadvantageous mutations such as those caused by kDNA integration in the course of Chagas disease is evidenced by the histopathology evident in the experimental rabbit and chicken heart tissues. We hypothesize that kDNA insertion is a mechanism triggering the parasite-independent autoimmune tissue-specific rejection in Chagas disease. Novel chimeric proteins encoded by ORFs formed by kDNA and host DNA juxtaposition have the potential to induce the immune response. The resulting phenotypic modification of mutated host cells could be a factor triggering autoimmunity in Chagas disease. This hypothesis represents a common denominator for explaining a long-lasting asymptomatic chronic infection in a majority of the patients, which could be correlated with harmless kDNA mutation in some chromosomal sites. Alternatively, it could explain the variable clinical manifestations, which

are characterized by remissions and exacerbations in some patients with mutagenic kDNA dispersal by active LINE-1 mobilization within the genome.

In the model presented here, kDNA insertion mutations could play different functional roles ranging from advantageous, neutral, and disadvantageous to the host. We cannot exclude the possibility of advantageous kDNA mutations that could associate emergence of adaptive characters that could be rapidly driven toward fixation by Darwinian natural selection (Klein and Takahata, 2002). The long-lasting, cryptic *T. cruzi* infection of vertebrate hosts is consistent with neutral mutations resulting from horizontal kDNA transfer and vertical inheritance in a vast number of progeny that could represent a prevailing mechanism of evolution at the molecular level. Neutral kDNA mutation, while having no necessary benefit to the host, can provide a substrate for natural selection. This type of mutation could play a major role in evolution mainly by loss, alteration, and refinement in certain groups of organisms subjected to environmental pressure over the evolutionary time scale. Crosskingdom molecular chimeras created by rearrangement and reshuffling of the host and pathogen genomes could be an unexplored force in speciation (Symer et al., 2002). In this respect, evolution is inherently opportunistic.

Randomly introduced kDNA mutation resulting from a persisting infection could explain the spectrum of Chagas disease manifestations seen 35 ± 10 years after the onset of vector-borne or congenitally transmitted *T. cruzi* in some human hosts (Lauria-Pires et al., 00; Schenone et al., 2001). The documentation and reproduction of horizontal transfer events from *T. cruzi* to the vertebrate host provide a solid foundation on which to continue our studies of Chagas disease pathogenesis. We are exploring the potential for expression of mutated host genomic DNA from specific integration events and examining the consequences of that expression at the molecular and organismal levels. Inflammation caused by the muscular disorders already appearing in chronic cases that have received kDNA integration through the germline of their parents (A.P. T., C. N.N., F.M., A.C.R., P. Gonçalves, and M.R. unpublished data). If these observations are validated, we have created a powerful new research tool for the understanding of chronic Chagas disease.

Experimental Procedures

Collection of Human Blood

We collected 10 ml of blood from street cleaners who volunteered to participate in this study. Serum samples collected were subjected to immunological testing for specific antibodies, and the blood-nucleated cells yielded DNA for biotechnological analyses (Lauria-Pires et al., 2000).

Growth of Parasites

The archetype Berenice *T. cruzi* (Chagas, 1909) and the β-galactosidase-expressing Tulahuen *T. cruzi* MHOM/CH/00 C4 (Buckner et al., 1999) were used. Trypomastigote forms of *T. cruzi* were harvested from murine muscle cell (L6) line and were cultivated in DMEM (pH 7.2) with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 250 nM L-glutamine at 5% CO_2 at 37°C. Epimastigote forms of *T. cruzi* were grown in liver-infusion tryptose axenic medium at 27°C. The *L. braziliensis* LTB300 stock (Evans, 1985) was grown in DMEM with 20% FBS. The parasite forms were harvested in the exponential growth phase.

Southern Blotting and PCR Analysis

Genomic DNA from uninfected controls; from *T. cruzi*-infected humans, chickens, and rabbits; and from *T. cruzi* and *L. braziliensis* in vitro cultures were used as templates for PCR with specific kDNA and nDNA sets of primers (Sturm et al., 1989; Moser et al., 1989). The mitochondrial kDNA was obtained from *T. cruzi* epimastigote forms and from *L. braziliensis* promastigotes, as described by Perez-Morga and Englund (1993).

Primers and Probes Used

The following primers and probes were used:

(1) S35 (5′-ATAATGTACGGG(T/G)GA TGC-3 6 (5′- TTCG ATTGGGGTTGGTG-3′).

(2) S34 (5′-ACACCAACCCCAAT AACC ; S67 (5 GTTTTGG GAGGGG(C/G)G/C)(T/G)TC-3′), DNA marker.

(3) Tcz1 (5′-CGAGTCCTT CCA GG-3′ z2 (5′-CCTCCA AGCAGCGGATAGTTC-3′ cz3 probe CGCTGCATCACA CGTT-3′), nuclear DNA r.

(4) kCR probe (5′- TGC TGGGAGGGGCGTTCAAATTTTGG CCCGAAAATTCATGCATCTCC CGTACATTATTTGGCCGAAAA TGGGGGTTGT TGGAGGTG GTTCGATTGGGGTTGGTGT AAG)represe a constant region of *T. cruzi* Berenice kDNA minicircle showing primers S34 d S67, respectively, at the extremities, and the nes S35 (Sturm al., 1989) antisense primer (underline) from 65 to 46 GenBan ccession number AF399841). The PCRs were carried ou plate DNA that was 20 times above the detection. In order to rule out differences in sensitivity of th echni the S35/S36 (Sturm et al., 1989) and Tcz (Moser et a 19 prim rs and probes, repeats were performed with up to 3 more nDNA and kDNA template. Each pair of primers (100 was used with 0.5 IU *Taq* and 0.2 mM dNTP in a 25 μL final vo e.

5′ RACE and Sequencing

The protocols for 5′ RACE used were according to Rudi et al. (1999). The PCR products were reamplified and directly cloned in pGEM T Easy vector. The clones were confirmed by hybridization using the kCR probe, and clones of interest were sequenced commercially.

T. cruzi Infections in Rabbits

Sexually mature New Zealand white rabbits were inoculated subcutaneously with 2.5×10^6 *T. cruzi* trypomastigote forms. Parasitemias in chronic *T. cruzi*-infected rabbits were shown by xenodiagnosis (Lauria-Pires et al., 2000). Rabbits showing positive parasitemias were bred for the study of the congenital transmission of the *T. cruzi* infections. The solid tissues from stillborn offspring and blood from surviving offspring were processed for DNA extraction (Sambrook and Russel, 2001). Tissue and blood from uninfected rabbits was processed identically.

Embryo Culture and *T. cruzi* Infections

Rabbit stem cell cultures were established from a blastocyst harvested 2 days postcoitus from a dam subjected to a Cesarean section under ketamine anesthesia. The fallopian tube was flushed with 5 ml of culture medium, and the zygotes collected in a dish were identified under an inverted microscope at 100× magnification, transferred individually to the wells of a 24-well plate, and kept at 5% CO_2, 37°C, in a humidified atmosphere. Six hours after incubation, the blast cells bursting out from the egg membrane adhered to the plastic surface and interacted with *T. cruzi* trypomastigotes. Two-day-old chicken embryo tissue was teased, suspended in DMEM complete medium, and transferred to 3 cm diameter petri dishes. Early embryo stage-derived embryonic stem cells growing at 37°C and 65% humidity were infected with β-galactosidase expressing *T. cruzi* trypomastigotes.

T. cruzi Inoculations in Fertile Chicken Eggs

Genetically selected White Ross chicken eggs that were obtained from Asa Alimentos (Recanto das Emas, Federal District) showed to be 86% ± 3% fertile. A 2 mm diameter hole was pierced in the

eggshell for injecting *T. cruzi* trypomastigotes in 10 μl of culture medium into the air chamber of stage X embryos. Mock controls received 10 μl of culture medium alone. Holes were sealed by adhesive tape, and *T. cruzi*-infected eggs as well as mock and uninfected control samples were incubated at 37°C and 65% humidity until hatching. The solid tissues from chicks that died after hatching and blood from surviving birds were processed for DNA extraction (Sambrook and Russel, 2001).

Inoculation of Naked kDNA in Rabbits and in Fertile Chicken Eggs

Four 60-day-old rabbits were injected with 375 μg of kDNA minicircles (Perez-Morga and Englund, 1993) or with 125 μg of a cloned kDNA sequence (GenBank accession number AF399842). In addition, 30 fertile chicken eggs received 15 ng of minicircles or 5 ng of cloned kDNA in the air chamber. The presence of kDNA in tissues was assayed by PCR.

Semen Collection for DNA Extraction

Semen collected from roosters (Penfold et al., 2001) was suspended in 1:10 volume of fresh DMEM and allowed to rest for 45 min at 5% CO_2, 37°C. Free-swimming sperm cells in the supernatant were harvested and subjected to DNA extraction (Sambrook and Russel, 2001).

Pathology and Immunochemical Analyses

The *T. cruzi*-infected chicken embryos and chicks that hatched from infected eggs were monitored daily for mortality. Tissues that were harvested from embryos and from chicks at set time were bisected so that half could be fixed in 0.02% glutaraldehyde (prepared in phosphate buffered saline [pH 7.2]) and stained with X-Gal (Buchner et al., 1999). X-Gal-stained tissues were then fixed in paraformaldehyde. Paraffin-embedded tissues were cut at 5 μm thick sections which were mounted by standard methods for microscopic examination. Sections showing blue cells were subjected to immunization with a Chagas diseased patient's serum (specific chemical anti-*T. cruzi* antibody 1:1024) and immunofluorescence staining with a fluorescein-conjugated rabbit anti-human IgG for colocalizing embryo cells harboring *T. cruzi*.

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Accession Numbers

The human-derived sequences (accession numbers AY490889 to AY490902, AY490904, AY490905, and AY485269), rabbit-derived sequences (AY488498 to 488503, AF400688, AF399841, and AF415293), and chick-derived sequences (AY237305, AY237306, and AY289115) have been deposited in GenBank.