Iranian J Parasitol: Vol. 6, No.3, 2011, pp.1-18

Iranian J Parasitol

Tehran University of Medical Sciences Publication
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Review Article

Genomic Organization of *Leishmania* Species

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(Received 12 Nov 2010; accepted 18 July 2011)

Abstract: *Leishmania* is a protozoan parasite belonging to the family Trypanosomatidae, which is found among 88 different countries. The parasite lives as an amastigote in vertebrate macrophages and as a promastigote in the digestive tract of sand fly. It can be cultured in the laboratory using appropriate culture media. Although the sexual cycle of *Leishmania* has not been observed during the promastigote and amastigote stages, it has been reported by some researchers. *Leishmania* has eukaryotic cell organization. Cell culture is convenient and cost effective, and because posttranslational modifications are common processes in the cultured cells, the cells are used as hosts for preparing eukaryotic recombinant proteins for research. Several transcripts of rDNA in the *Leishmania* genome are suitable regions for conducting gene transfer. Old World *Leishmania* spp. has 36 chromosomes, while New World *Leishmania* spp. has 34 or 35 chromosomes. The genomic organization and parasitic characteristics have been investigated. *Leishmania* spp. has a unique genomic organization among eukaryotes; the genes do not have introns, and the chromosomes are smaller with larger numbers of genes confined to a smaller space within the nucleus. *Leishmania* spp. genes are organized on one or both DNA strands and are transcribed as polycistronic (prokaryotic-like) transcripts from undefined promoters. Regulation of gene expression in the members of Trypanosomatidae differs from that in other eukaryotes. The trans-splicing phenomenon is a necessary step for mRNA processing in lower eukaryotes and is observed in *Leishmania* spp. Another particular feature of RNA editing in *Leishmania* spp. is that mitochondrial genes encoding respiratory enzymes are edited and transcribed. This review will discuss the chromosomal and mitochondrial (kinetoplast) genomes of *Leishmania* spp. as well as the phenomenon of RNA editing in the kinetoplast genome.

Keywords: *Leishmania*, Kinetoplast, Genome, RNA editing, Trans-splicing

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Introduction

Leishmania spp. lives in the gastrointestinal tract of the sand fly vector, and can be cultured using appropriate laboratory culture media as promastigotes. They can also exist in the vertebrate host macrophages in the amastigote form (1-3). While asexual reproduction is known to occur in this species (4), their sexual forms have not yet been discovered (5). Clonal reproduction is believed to occur among the protozoan parasites of the family Trypanosomatidae (6), considering that nuclear fusion occurs in some forms of this parasite that may give rise to sexual reproduction (7, 8). Researchers have been unable to confirm sexual reproduction and identify sexual gametes of these microorganisms by using classical methods (9). It should be noted that the exchange of genetic material in Trypanosomatidae has been proven (10-12).
Leishmania is used as an intracellular molecular model for research in microbiology, immunology, and biochemistry (1, 2, 13-20). This article will discuss the genomic organization of this parasite.

Genomic organization of *Leishmania*

The haploid genome of *Leishmania* spp. has 32,816,678 bp organized into 36 chromosomes (21), with a total of 911 RNA genes and 39 pseudo-genes (21, 22). A total of 8272 genes are known to encode proteins. Producer protein genes are encoded as long polycistronic genes lacking transcription factors in *L. major*, *Trypanosoma brucei*, and *T. cruzi* (Tritryp) (Fig. 1). The Old World *Leishmania* spp. has 36 chromosomes, while the New World *Leishmania* spp. has 34 or 35 chromosomes. *L. mexicana* has linkage groups of chromosomes 8 and 29 as well as of chromosomes 30 and 36, and *L. braziliensis* has a linkage group of chromosomes 20 and 34 (23). The general pattern of nucleotide sequences of genes in 30 *Leishmania* spp. is conserved (24-26).

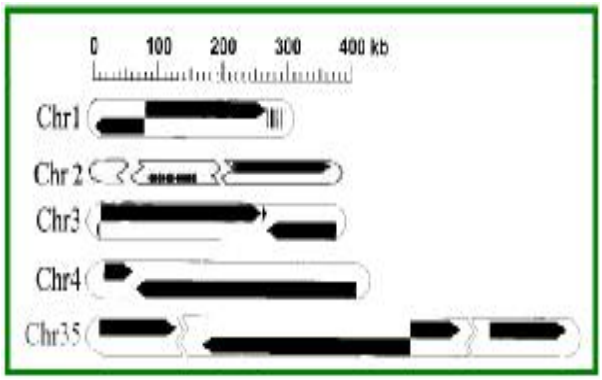

Fig. 1: Organization of chromosomes of *Leishmania* genes: clusters of genes on chromosomes 1, 2, 3, 4, and 35 are shown as thick lines. The direction of mRNA transcription is indicated. Vertical lines indicate the right side of the chromosome 1 repeated sub-telomeric sequence. The arrows indicate chromosome 2 splice leader categories. The arrows between the individual genes in chromosome 3 genes indicate tRNA. The space on chromosome 35 indicates an area of undetermined sequence (Source Ref. 30).

Chromosome 1 of *L. major* is the smallest *Leishmania* spp. chromosome, and contains 79 protein-encoding genes. Its genes have been organized into 2 converted polycistronic clusters, and mRNA transcription is directed to the telomeres (27-30).
Leishmania spp. chromosome 3 has about 79 genes, and is organized as 2 convergent polycistronic transcripts. These transcripts encode 2 protein clusters and tRNA genes are located between them. They remain at the end of a gene that is transcribed in contrast to the previous clusters (28, 29, 31).
Leishmania spp. proteins are expressed during translation or after completion of replication (32). The mechanism involves regulation of transcription of the eukaryotic RNA polymerase II. This mechanism differs from

the other mechanisms, although they have a chromatin remodeling process (21).

In contrast to other members of Trypanosomatidae, the *Leishmania* genome does not have a sub-telomeric region (species-specific genes) and a transposable element. There have been no reports of RNAi in this organism (22).

The *Leishmania* spp. genome is organized in the nucleus, which contains chromosomal and episomal DNA, and in the kinetoplasts, which comprise independently replicating DNA molecules. Furthermore, virus-like particles are contained in the cytoplasm. The kinetoplasts have been separated and studied by ultracentrifugation, whereas the chromosomes have been studied by pulsed-field gel electrophoresis (PFGE). There are questions regarding the changes occurring in karyotype species, sexual reproduction in *Leishmania* spp., and the number of copies of each gene in each chromosome (33).

The electrophoretic patterns of *Leishmania* spp. chromosomes investigated by PFGE indicate haploid, diploid, and polyploid arrangements. Isolation by hybridization that parts of chromosomes can be common, but the genes Hsp70, Hsp80, adenylate cyclase, glyceraldehyde phosphate dehydrogenase, beta tubulin, phosphofructokinase, phosphoenolpyruvate carboxymethyl pyruvate kinase, pyruvate kinase, and ubiquitin are conserved. The chromosomes range from 400 to 900 kbp in size and contain mini-exons (5′-spliced leader genes). Chromosomal changes that occurred during the evolution of *Leishmania* spp. have been confirmed, and the molecular karyotypes in the promastigote and amastigote forms have been found to be identical. Three molecular karyotypes have been identified in *Leishmania* spp.: (1) The *L. major* karyotype is completely conserved, even in different geographical regions, (2) The members of the *L. braziliensis panamensis* group have more than one karyotype, and (3) *L. mexicana amazonensis* has highly diverse molecular karyotypes, even among those isolated from the same clinical samples. The mechanism of chromosomal polymorphism in *Leishmania* spp. does not include removal or translocation. Among the genes amplified in *Leishmania* spp. are genes that confer resistance against sodium arsenate and methotrexate drugs. This phenomenon of increasing the number of copies of genes involved in metabolic phenomena and environmental response appears to be important. Regions of genes that are involved in drug resistance are increased by 2–20 folds in copy number. Two genomic regions, namely, H-DNA and R-DNA (encoding dihydrofolate reductase and thymidylate synthase), are chromosomal derivatives, which are surrounded by inverted repeats. The inverted repeats are involved in the supercoiling of amplified gene products (33).

Methotrexate and arsenate drugs induce gene amplification in *Leishmania* spp. Methotrexate induces amplification of the R-DNA and H-DNA genomic regions. In a methotrexate-resistant *L. tarentolae* mutant, the H region is amplified as linear or circular DNA. The dihydrofolate reductase, thymidylate synthase, and ltdh genes in the H region are resistant to drugs (34, 35). Gene amplification in the amphotericin B-resistant *L. tarentolae* occurs in the circular form in different chromosomes (36). Drug resistance to sodium stibogluconate (pentostam) in *L. tarentolae* is due to the amplification of a gene described by Haimeur and Ouellett, which encodes a 770-amino acid-long protein (37).

Gene expression control among the members of the parasitic Trypanosomatidae family involves unusual antigenic shifts, involving DNA rearrangements, generation of polycistronic transcripts from multi-copy genes, and post-transcriptional modification by trans-splicing and RNA editing (38).

Gene transcription in *Leishmania* spp.

The genetic information of most organisms has been discovered in cDNA sequences known as expressed sequence tags (EST) (39). It should be noted that most *Leishmania* genes have no introns (40), and that chromosomal DNA is used as the template for cloning by PCR (41-45).

Gene transcription to produce proteins in eukaryotes involves RNA polymerase II and transcription by RNA polymerase I to produce ribosomal RNA. In kinetoplastids, gene transcription involves RNA polymerase I and a trans-splicing mechanism (46).

Discontinuous mRNA synthesis is a process occurring in the kinetoplastids. In this process, a 35-nucleotide sequence is placed at the 5′-end of all mRNAs. This sequence is encoded by a gene duplication cluster, 1.35 kb in length, which is known as a mini-exon or a trans-splice. Mini-exon mRNA was first identified as being related to the trypanosome variable surface glycoprotein (47).

Martınez-Calvillo et al. analyzed the sequence of *Leishmania* chromosome 1, which is the smallest of all the chromosomes. A total of 39 genes were transcribed from a strand of DNA and 50 other genes in a polycistronic transcript (48). Martınez-Calvillo et al. also analyzed *Leishmania* chromosome 27 and indicated that the organization of transcription of *Leishmania* genes is a complex process. It was determined that chromosome 27 of *Leishmania* spp. is transcribed by RNA polymerase II (49). This is contrary to the findings of Ploeg and Lee (46).

Non-coding RNAs, about 300–600 nucleotides long, are known to be expressed only in the amastigotes; these RNAs are transcribed by RNA polymerase II. Both sense and antisense transcripts are processed by trans-splicing and polyadenylation, but the antisense transcripts are transcribed 10 folds lesser than the sense transcripts. It is possible that these antisense transcripts play a role in RNA stability. It should be noted that these molecules are not transcribed in promastigotes, and that RNA stability in promastigotes is less than in amastigotes (50).

Trans-splicing of the *Leishmania* mRNA transcript

There is a 35-nucleotide-long sequence known as a spliced leader (SL) or 5′-mini exon at the 5′ end of *Leishmania* mRNA transcripts. The SL sequence is at the 5′-end of a preliminary transcript about 85 nucleotides in length that contains a 5′-exon-intron connection adjacent to the 3′-spliced leader (Fig. 2 and 3).

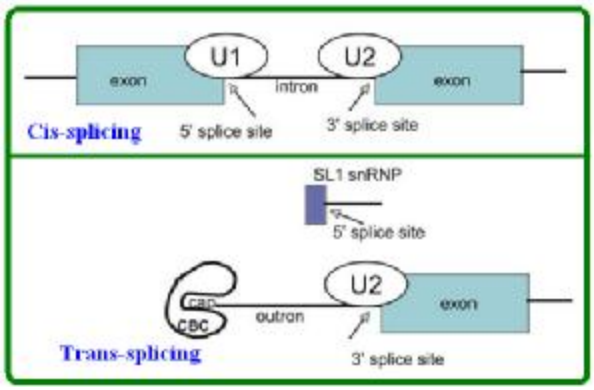

Fig. 2: Comparison of cis- and trans-splicing: In cis-splicing, pair bases U1 small nuclear ribonucleoprotein (snRNP) are in the 5′ SL [?] and U2 snRNPs are in the break point, while intron breaks two exons are connected. In trans-splicing, a 5′-splice site on the mRNA for binding to U1 snRNP is absent. Instead, a 5′-splice site produced by the donor SL snRNP interacts with U2 in the 3′-splice site. The splice leader connects to the next exon. (http://www.wormbook.org/chapters/www_transsplicingoperons/transsplicingoperons.pdf)

The sequence of the SL connects the 3′-end of the genes encoding proteins. Previous reports have indicated the possibility of such intermediaries in mRNA processing. There is a 50-nucleotide-long interval at the 3′-end of SL, which is known as the SL intron se-

quence (SLIS). The SLIS and SL are connected to the 5′-end of RNA. Density centrifugation analyses have shown that SL mRNA is in the 60S rRNA, but SLIS is in the 40S rRNA. It is likely that the observed nucleoprotein particles are the same spliceosomes that can be observed in other microorganisms (51, 52).

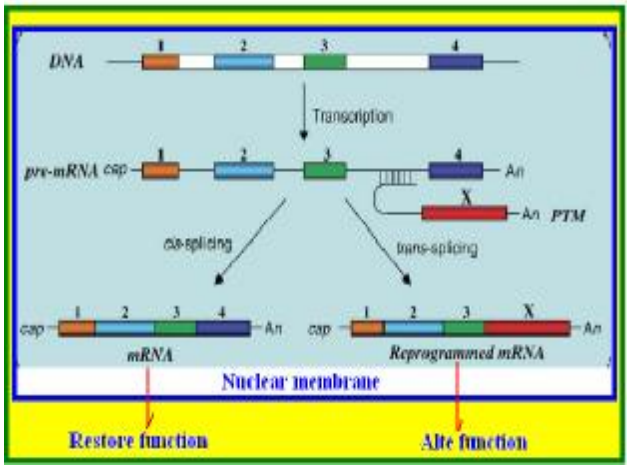

Fig. 3: Cis-splicing and trans-splicing: There are 4 exons in the initial transcript, which contains both exons and introns. In the cis-splicing phenomenon, the mRNA contains 4 exons and 3 introns. The 3 introns are removed, and the exons are connected. In trans-trans-splicing, a pre-trans-splicing molecule attaches exon X to intron 3. The 5'-splice donor is attached to the 3'-splice acceptor (Source Ref. 52).

Since the discovery of trans-splicing in *Leishmania* spp., it has also been observed in other microorganisms (53). Trans-splicing is an essential stage of eukaryotic precursor mRNA and is not observed in mammals, insects, yeast, and plants (54). This phenomenon is observed in rotifera (55), dinoflagellates (56), nematodes, and protozoan parasites (57-60) as shown in Fig. 4.

Table 1: Comparison of the characteristics of the genomes of 3 species of *Leishmania* (22)

Leishmania spp. Characteristics	*L. major*	*L. infantum*	*L. braziliensis*
Chromosome	36	34	35
Contigs	36	562	1041
G + C percent	89.7	59.3	57.76
Size (No nucleotide; bp)	32,816,678	32,134,935	32,005,207
Coding genes	8298	8154	8153
Pseudo genes	97	41	161
G + C content (%) in codir region	52.5	52.45	60.38

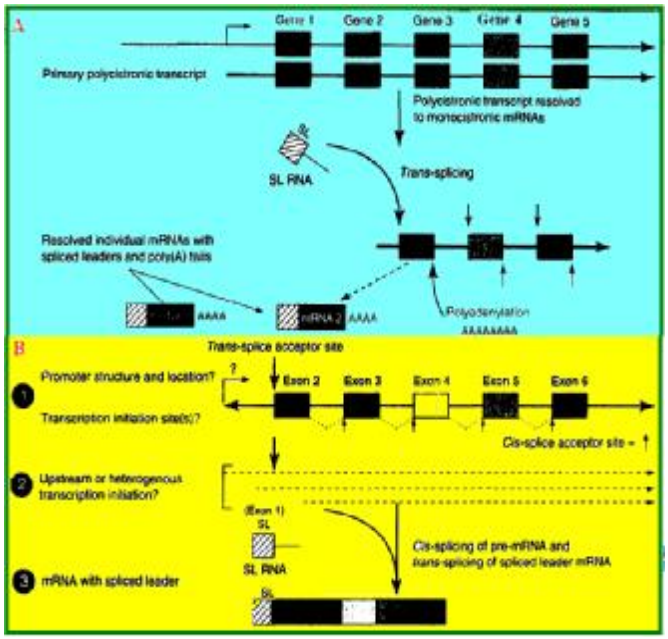

Fig. 4: Trans-splicing in metazoan parasites: A) Transcription occurs via a polycistronic transcript and trans-splicing. The initial transcript contains mRNAs with 5′-trans-splicing and polyadenylation. Each box represents 1 gene with an exon and an intron. The bent arrows indicate the promoter and the transcription start site. B) The phenomenon of transcription and trans-splicing in metazoan genes (worms). The solid squares indicate genes with an intron between them. 1) Promoter and possible transcription start site. 2) The position of transcription initiation. 3) mRNA molecules with SL (Source Ref. 60).

The organization and regulation of gene expression in trypanosomatid parasites differs from that of other cells. Collected information has led to advances in effective disease control (61).

The genes in the parasites of family Trypanosomatidae are organized as long polycistronic transcripts (more than 100–300 kb) on the same DNA strand. The genes encoding proteins are transcribed from unknown promoters, and precursor polycistronic RNA is produced. Monocistronic mRNA is produced by trans-splicing and polyadenylation of RNA. The trans-splicing mechanism includes a mini-exon containing 39 nucleotides, which is not translated. This mini-exon is connected to the 5′-end of the mRNA molecule. There are some similarities between cis-splicing and trans-splicing mechanisms. The AG is at the 3′ of the splice acceptor site downstream of a polypyrimidine tract. Polyadenylation in *Leishmania* spp. requires trans-splicing and differs from that of other eukaryotes. There are no introduced polyadenylation signals in kinetoplastidae undefined and instead choose to place poly A site depends on positions upstream acceptor site (61.) Gopta et al. analyzed chromosomes 1 and 3 of *L. major* and predicted the positions of trans-splicing with 92% accuracy. Computer analyses were performed to identify elements involved in trans-splicing. The following components are present: (1) nucleotide A, (2) a polypyrimidine rich stretch of T and C, varying in size from 5 to 100 nucleotides with purine bases occasionally located between

the T and C, (3) a variable spacer, and (4) a 3′-acceptor site consisting of AG (62).

Synthesis of nucleic acids in *Leishmania* spp.

Leishmania spp. generates pyrimidine nucleic acids via *de novo* biosynthesis, but obtains purine nucleic acids via a salvage process (63-66).

Cunningham and Beverley have studied the amastigote stage of pathogenic species of *Leishmania*. This study indicated that salvage activities in the amastigote stage would limit the effectiveness of chemotherapy in patients infected with *Leishmania* spp. Salvage activities do not involve RNA transcripts and likely occur via posttranscriptional modifications (67).

Structure of the kinetoplast

The kinetoplast or mitochondrion is the energy-producing organelle of *Leishmania* spp. (68, 69). If DNA replication is inhibited in the kinetoplast by ethidium bromide, energy production will reduced in the parasite (70).

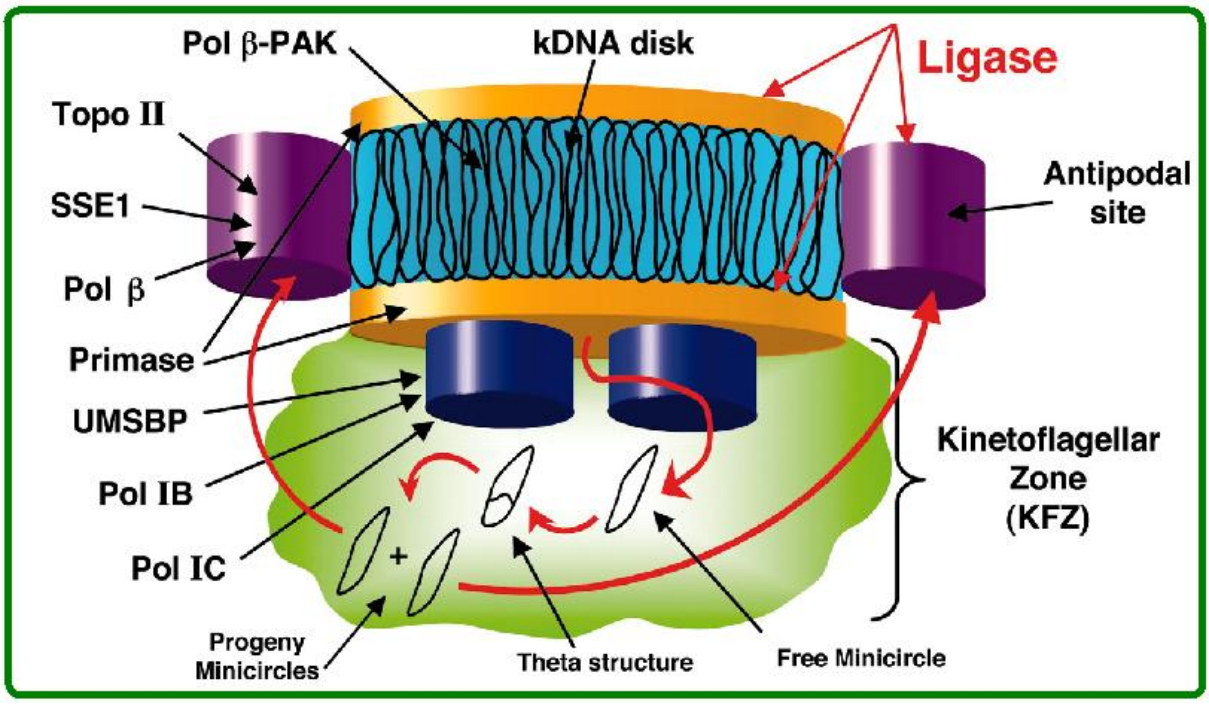

Fig. 5: Structure of the kinetoplast disk and the proteins involved in its replication
SSE1, Structure -specific endonuclease 1; UMSBP, Universal minicircle sequence-binding protein
(http://www.pnas.org/content/101/13/4333/F2.expansion.html)

The extra-chromosomal DNA is located in the kinetoplast organelle in an arrangement similar to that in the mitochondria of the eukaryotes. The kinetoplast has a particular DNA topology, which is not found in other eukaryotic cells (Fig. 5), and is composed of large circular molecules up to about 50,000 nucleotides that are known as maxicircles. They are not present in large numbers. These circular molecules carry the genes encoding

the enzymes and coenzymes involved in the Krebs cycle (71-74).

Other DNA molecules that are present in larger numbers but have fewer nucleotides (600–2,500 bp) are known as minicircles. *Chritidia fasciculate* has 25 maxicircles (each containing 37,000 nucleotides) and 5,000 minicircles (each containing 2,500 nucleotides) (72-74), some of which have been identified as free-form molecules (73). A minicircle DNA sequence "GGGGTTGGTGTAA" is conserved among all members of the family Kinetoplastidae (75), and some believe that this sequence is the origin of replication of the minicircle (76). Other parts of the minicircle sequence vary among the minicircles. One region known as the variable region is used for parasite genotyping. Large and small circles can exist inside each other so that each loop intercalates with 2 other minicircle loops and eventually "maxicircles and minicircles" become intertwined with each other (interlocked or catenated) and a heavy molecule (about 400S) is formed during the extraction of parasite DNA that is distinct from the chromosomal DNA sediment. When minicircles replicate, some are released (Fig. 6) as opened loops. When replication is completed, a replicated minicircle will become connected to the kinetoplast (75, 77, 78). The blank section of the kinetoplast is restored by DNA topoisomerase II (79).

The origin of replication of the kinetoplast is recognized by a protein known as UMSBP (universal minicircle sequence-binding protein). This protein is responsible for initiating replication (80). This reaction is regulated *in vivo* by an oxidation-reduction reaction (80, 81). The inhibition of UMSBP halts the growth of the parasite (82). A zinc ion (Zn) is involved in this process, and is essential for connecting UMSBP to DNA (83). It should be noted that replication of the members of Kinetoplastidae occurs via different mechanisms (84).

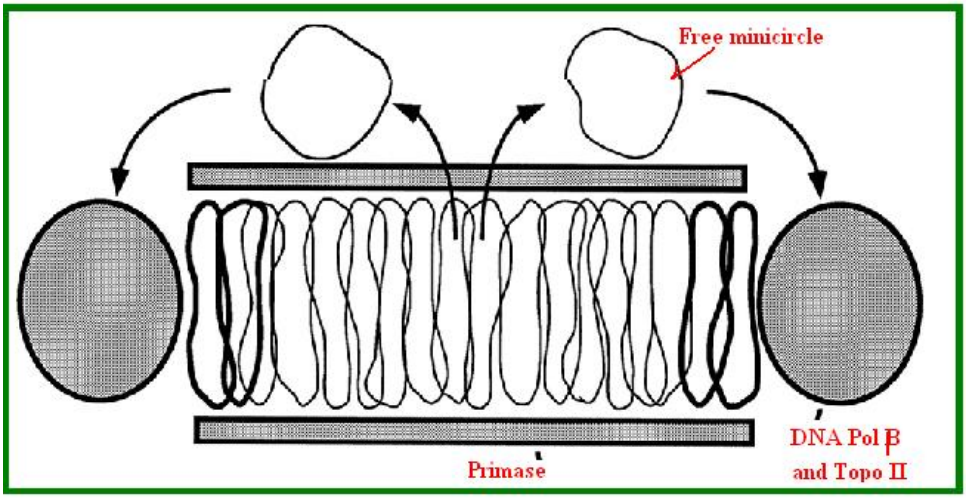

Fig. 6: *In vivo* replication of a kinetoplast shown as a disk section with catenated minicircles surrounded by DNA polymerase beta and DNA topoisomerase II. Primase is located at the top and bottom. During replication, the minicircles are released and connected to the network after replication is complete. Two newly synthesized minicircles are shown in bold (http://www.jbc.org/content/272/33/20787.full.pdf+html)

The functions of minicircles were not clarified until recently, and the genes of some of the enzymes involved in Krebs cycle were not observed. The discovery of the RNA

editing phenomenon was an exciting new finding (85-87). It was found that the parasites have copies of RNA molecules, which are altered because of posttranscriptional modification. This is accompanied by deletion or insertion of a number of nucleotide residues (mostly uracil). RNA editing emits signals by guide RNA-derived transcripts of minicircles (88, 89) or maxicircles (90) (Fig. 7). A gRNA-binding complex is involved in the processing of a gRNA, which includes polyadenylation and stabilization of the edited mRNA transcript (89). The kinetoplast of *L. tarentolae* has a 9S rRNA (91-94) and a 12S rRNA (92-95), but does not have supercoiled circles (96).

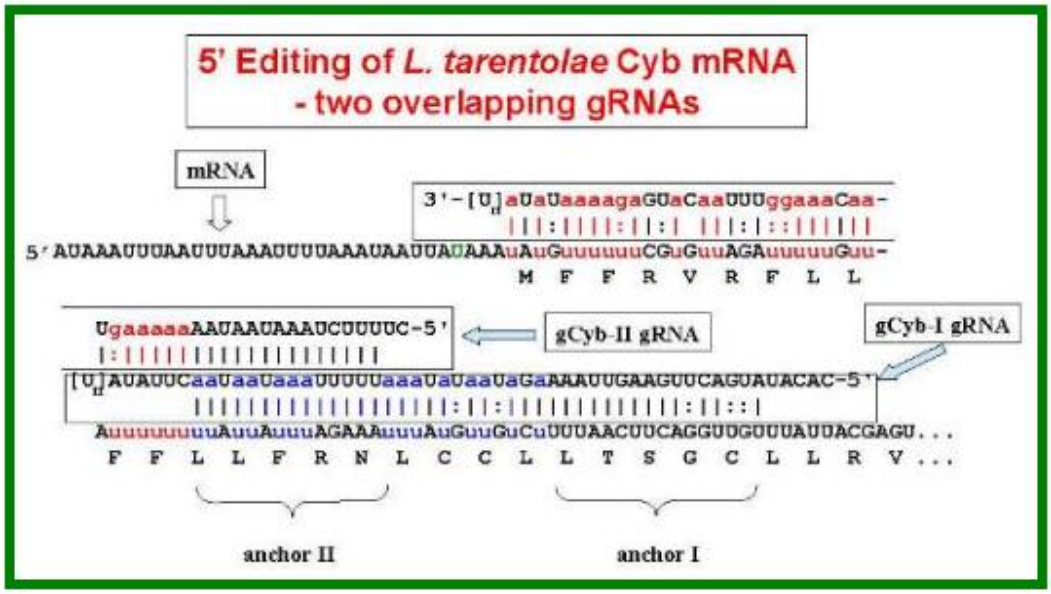

Fig. 7: RNA editing of cytochrome oxidase B of *Leishmania tarantula* (http://dna.kdna.ucla.edu/trypanosome/index.html)

The RNA editing phenomenon produces deletions, replacements, and insertions in mRNA transcripts (86, 87, 97). An edited transcript mRNA has important effects, and sometimes half of the nucleotides are altered (Fig. 8). It should be noted that although the changes may be small, its effect is important. For example, replacement of a C nucleotide by U in the human apolipoprotein B transcript (Fig. 9) leads to conversion of a glutamine codon to a stop codon. This edited transcript produces a truncated protein (85).
The TGA codon (stop codon) of the *Leishmania* spp. maxicircle encodes tryptophan (98). When the parasite glycosomal cycle is reduced, the mitochondrial (kinetoplast) volume is increased, and vice versa (99). The characteristics of kinetoplast DNA has led to its choice as a target for drug therapy (99, 100).

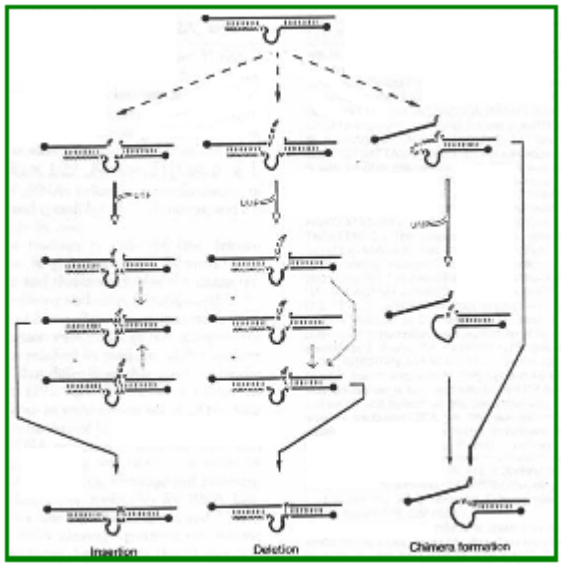

Fig. 8: Model RNA editing in the kinetoplast: addition of U (left), removal of U (center) or formation of a chimera (right) in an mRNA transcript are performed by TUTase (http://dna.kdna.ucla.edu/trypanosome/images/kablea.JPG)

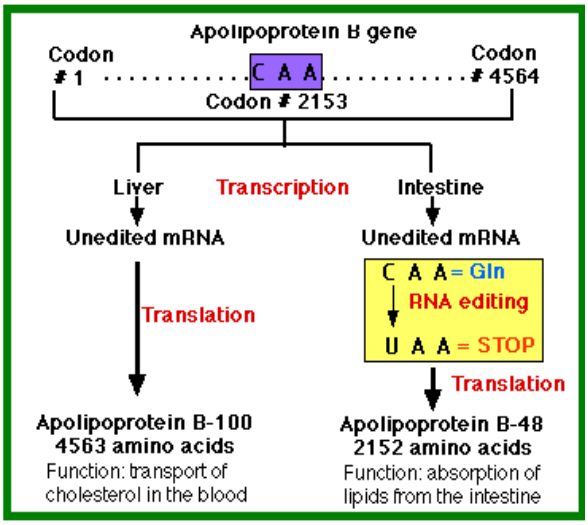

Fig. 9: RNA editing in human apolipoprotein B

Discussion

Leishmania is a protozoan parasite with some similarities and differences as compared to other eukaryotic cells. It shares some characteristics with prokaryotic cells, such as polycistronic transcription (31, 38). Researchers have been attracted to its unique characteristics. In recent years, *Leishmania* spp. has been used as a host for production of recombinant proteins. An appropriate host is an important factor in production of recombinant proteins (drugs). Prokaryotes such as *Escherichia coli* need simple and inexpensive culture media and have a short proliferation time. This provides high yields in the production of recombinant proteins. However, prokaryotes do not generate post-translational modifications such as glycosylation, phosphorylation, and car-boxylation. Some eukaryotic proteins are non-functional after translation in *E. coli*, and some of them become aggregated as inclusion bodies in the host cell cytoplasm. These proteins cannot fold appropriately if they are expressed in a prokaryotic host.

Replication of yeasts such as *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* also requires significant culture time, and posttranslational modifications are not perfect processes. Other types of eukaryotic cell cultures tend to be expensive and require specialized culture conditions and laboratory equipment. Because *Leishmania* spp. is maintained easily in NNN culture medium at low cost and can multiply quickly, it is preferred over other species. Eukaryotic *Leishmania* spp. can perform posttranslational modifications (21). This makes *Leishmania* spp. a suitable host for production of recombinant protein drugs (101-104). Efforts undertaken thus far have allowed the production of some therapeutic proteins (105-108). However, more research is needed before it can be used extensively as a host for the production of recombinant proteins. Researchers in biochemistry, pharmacology, and immunology, who are engaged in new drug development as well as production and testing vaccines, need

appropriate cell models. *Leishmania* spp. is expected to be an appropriate candidate (109,110). Anti-folates are used to treat malaria, bacterial infections, and cancer. *Leishmania* spp. is an appropriate model for testing such enzyme inhibitors to investigate the progression of anti-parasitic and anti-cancer drugs (111, 112). Posttranslational modifications play important roles in various cellular processes. Small ubiquitin-like modifier protein (SUMO) is a fusion protein that can be added as a reversible tag to N terminal recombinant proteins (eukaryotes and prokaryotes) to provide stability and solubility to proteins (113, 114). SUMO is produced by *Leishmania* spp. and can be used as a stable and soluble factor for the production of recombinant proteins in *Leishmania* promastigote (115). One disadvantage is that non-coding RNAs act as mRNA stability factors are not transcribed in the *Leishmania* promastigote. Because mRNA is not stable in promastigotes (50), this form of *Leishmania* spp. is not appropriate for use as a host in the preparation of recombinant proteins.

Glossary

Biosynthesis de novo: The de novo purine biosynthetic pathway produces purines which represent the building blocks for DNA and RNA synthesis

Cistron: A segment DNA equivalent to gene for function (protein or enzymes)

Diploid; An organism with sexual cycle is diploid and has one chromosome set from each of its parents

Haploid: The haploid means usual number of chromosomes set in somatic cells of common organisms. Organisms that have not sexual cycle are haploid

Inversion: Chromosome break of the two areas separated pieces is back by reversal from chromosome breakage

Inverted repeats: Is a sequence of nucleotides that is the reversed complement of another sequence further downstream

Poly cistronic: There are some cistrons on one mRNA

Polyploidy: Increase in chromosome set number

Pseudo-genes: Are copy of original gene sequence, but lacked the necessary sequences for function. These genes from genetically similar to functional genes, but they have containing multiple mutations

snRNP: Small nuclear ribonucleoproteins, are RNA-protein complexes, they will combined with unmodified pre-mRNA and various other proteins to spliceosome formation.

Sub telomeric: Sub telomeric is a region near the end of chromosomes composed of polymorphic repetitive DNA. Damage to this area in humans lead to mental retardation

Acknowledgements

The author thanks the authorities of the Cellular and Molecular Biology Research Center of Shahid Beheshti University of Medical Sciences for their collaborations and declares that there is no conflict of interests.

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