Formation of linear inverted repeat amplicons following targeting of an essential gene in *Leishmania*

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ABSTRACT

Attempts to inactivate an essential gene in the protozoan parasite *Leishmania* have often led to the generation of extra copies of the wild-type alleles of the gene. In experiments with *Leishmania tarentolae* set up to disrupt the gene encoding the J-binding protein 1 (*JBP1*), a protein binding to the unusual base \(\beta\)-\(D\)-glucosyl-hydroxymethyluracil (J) of *Leishmania*, we obtained *JBP1* mutants containing linear DNA elements (amplicons) of \(\sim 100\) kb. These amplicons consist of a long inverted repeat with telomeric repeats at both ends and contain either the two different targeting cassettes used to inactivate *JBP1*, or one cassette and one *JBP1* gene. Each long repeat within the linear amplicons corresponds to sequences covering the *JBP1* locus, starting at the telomeres upstream of *JBP1* and ending in a \(\sim 220\) bp sequence repeated in an inverted (palindromic) orientation downstream of the *JBP1* locus. We propose that these amplicons have arisen by a template switch inside a DNA replication fork involving the inverted DNA repeats and helped by the gene targeting.

INTRODUCTION

*Leishmania* sp. is a unicellular protozoan parasite belonging to one of the oldest eukaryotic lineages, the Kinetoplastida. *Leishmania* is known for its genomic plasticity. Changes in the karyotype of *Leishmania*, such as triploidy for one chromosome or genome-wide polyploidy, have been reported following attempts to inactivate essential genes (1–3). DNA amplification can also be induced in this parasite by selection with a variety of drugs including methotrexate (4–7), oxyanions (antimony, arsenite) (8–11), mycophenolic acid (12), tunicamycin (13) and vinblastine (14). Amplicons have even been found in unselected laboratory isolates (15).

The structure of the amplicons in *Leishmania* varies: they can be circular or linear, and can contain direct or inverted DNA repeats. They are usually the product of a conservative amplification with no alteration in the source chromosome and are frequently lost in the absence of the selection that induced the amplification (16). The preferred model of DNA amplification in *Leishmania* resulting in inverted DNA repeats involves the self-annealing of inverted (palindromic) DNA repeats in a region of single-stranded DNA being replicated, followed by the self-replication of one of the DNA strands and the consecutive synthesis of its complementary strand (5,9,17). Whereas in mammalian cells DNA amplicons are often the product of many successive rearrangements (18), DNA amplification in *Leishmania* normally occurs in one step. Hence, the amplicons found in *Leishmania* usually have a simple structure, which makes this parasite a good model for the study of gene amplification (16).

Base J or \(\beta\)-\(D\)-glucosyl-hydroxymethyluracil is a unique hypermodified base only present in the nuclear DNA of kinetoplastid parasites and *Euglena* (19–21). It replaces \(<0.5\%\) of thymine in the genome of *Trypanosoma*, mostly in repetitive sequences (22), including the telomeric repeats (20). The enzymes involved in J biosynthesis have not been isolated yet. Hence, we have not been able to produce parasites without J and the function of J remains a matter of speculation. We isolated a J-binding protein, however, named JBP1 for J-binding protein 1 (23), which binds to J-containing duplex DNA with high specificity (24,25). *JBP1* null trypanosomes are viable *in vitro* and in mice, and have no obvious defect in the stability of their DNA repeats or in gene expression.

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Interestingly, they have 20-fold less J, suggesting that JBP1 is involved in the maintenance of J levels in *Trypanosoma brucei* (26).

To get more insight into the function of JBP1, we tried to inactivate the *JBP1* gene by gene targeting (via homologous recombination) in the promastigote (insect) form of *Leishmania tarentolae*, a parasite of lizards. We could easily inactivate one allele, but all attempts to make a *JBP1* null *Leishmania* resulted in cell lines maintaining a wild-type allele, suggesting that *JBP1* is essential in this parasite. Some of these lines were found to contain new linear DNA plasmids, harboring the targeting constructs used to inactivate *JBP1*. We present here the structure of these plasmid amplicons and a model for their generation during the targeting of the *JBP1* locus.

**MATERIALS AND METHODS**

**Culture cell lines and transfections**

The *L. tarentolae Tar1WT* cell line (5) was cultured in SDM 79 medium (27). Transfections were done as described previously (6). The cells were selected at the following drug concentrations: 20 μg puromycin (Sigma) per ml; 20 μg neomycin (Gibco) per ml; 100 μg hygromycin B (Roche) per ml; 200 μg paromomycin (Sigma) per ml. The frequency of transfection was determined by serial dilution of the cells after transfection. The reversion experiment was done by culturing the amplicon-containing cell lines in the absence of drug pressure. The DNA measurement by flow-cytometry was done as described in Munoz-Jordan and Cross (28).

**Cloning procedures and inactivation constructs**

The cloning of the *L. tarentolae* *JBP1* gene was already described in Cross et al. (23). A cosmid containing *L. tar JBP1* was isolated by screening a cosmid library reported in Brochu et al. (29), and a ~7.2 kb fragment containing *JBP1* was digested with HindIII–ClaI and cloned in pBluescript (Stratagene) giving the construct *Ltar JBP1*-pBluescript. To make the KO NEO and KO HYG inactivation constructs, the NEO and HYG markers, both preceded by a ~90 bp polypyrimidine stretch (Y), were first digested out of the PSPY-NEO and PSPY-HYG vectors (30) by a XbaI–BglII digest, and cloned in PSL1180 (Amersham) giving, respectively, the PSLY-NEO and PSLY-HYG vectors. A ~2.4 kb Apal fragment covering the 3′-untranslated region (3′-UTR) part of *Ltar JBP1* was isolated by an Apal digest of *Ltar JBP1*-pBluescript, and cloned downstream of the NEO or HYG markers by insertion in the Apal site of PSLY-NEO or PSLY-HYG. A ~2 kb HindIII–XbaI fragment covering the 5′-UTR of *Ltar JBP1* was then cloned into the HindIII–SpeI digested PSLY-NEO + 3′-UTR or PSLY-HYG + 3′-UTR vectors, leading to the *Ltar JBP1 KO NEO* and *KO HYG* constructs. The cassettes were taken out of the PSL1180 backbone by a HindIII–BstBI double digest prior to transfection. Note that the NEO marker used by us has a point mutation at position 406 (bp) (C → A mutation, Gly → Lys). To construct the KO PUR inactivation cassette, the gene coding for the puromycin acetyltransferase was HindIII–ClaI cloned after the second α-tubulin intergenic region (containing processing signals) of pGEM TZf α-NEO-α-PUR. The α-PUR cassette was then subcloned into the EcoRI–ClaI sites of pSP72 (Promega), and finally integrated between the 5′-UTR and 3′-UTR sequences of *JBP1* by an XbaI–BglII digest of the *Ltar JBP1 KO NEO* construct (which removes the YNEO cassette). The *Ltar JBP1 KO PUR construct* was linearized with Ncol prior to transfection. The rearrangement point of the *JBP1* KO NEO amplicon was cloned by ligation ~2.0 kb EcoRV fragments and ~3.0 kb BamHI fragments of digested genomic DNA of *JBP1* KO NEO into pBluescript (i.e. fragments of the size of the rearrangement point for these digests). The clones of interest were isolated by screening with the probe 9. Similar experiments were done in order to clone the rearrangement point of the *JBP1 KO HYG* construct and KO PUR:KO HYG amplicons. The sequences corresponding to the rearrangement point in the *Tar1WT* cell line were isolated following similar procedures.

**DNA extraction, blotting and hybridization**

DNA was gel-extracted using a QIAGEN gel-extraction kit. DNA electrophoresis and blotting were performed using standard conditions (31). Membranes were hybridized with radioactively labeled probes using the Prime-it random primer labeling kit (Stratagene) at 42°C in a formamide buffer [48% formamide, 5× SSC (0.75 M NaCl and 75 mM sodium citrate, pH 7.0), 10× Denhardt’s (1% Ficoll (Pharmacia), 1% polyvinyl pyrrolidone and 1% BSA (Roche)), 50 mM NaCl (Na2HPO4, NaH2PO4), 5 mM EDTA, 0.1% SDS and 0.25 mg salmon sperm DNA per ml]. Membranes were washed with 3× SSC, 0.1% SDS and 0.1× SSC and 0.1% SDS. The *JBP1* 3′-UTR probe corresponds to the ~2.4 kb Apal fragment described in the Cloning section. The XbaI–BglII YNEO and YHYG cassettes (see Cloning section) were used as probes to check for the site of integration of the constructs. The telomeric probe was described in van Leeuwen et al. (20). Hybridization with the telomeric probe was done at 65°C in a water-based buffer [6× SSC, 5× Denhardt, 0.02% SDS, 2 mM EDTA, 20 mM NaPi (pH 7.4) and 0.1 mg tRNA per ml]. Washes were done with 6× SSC and 0.1% SDS.

**Chromosome separation and amplicon extraction**

DNA plugs were made as described in van der Ploeg et al. (32). Chromosomes were separated using a CHEF-DRII apparatus (Bio-rad) under the following conditions: 24 h, 6 V/cm, initial switch time: 35 s, final switch time: 120 s. Amplicons were extracted from low melting point agarose gels by agarase (New England Biolabs) treatment following the indications of the manufacturer.

**Sequencing**

The cosmid covering the *JBP1* locus was sequenced by shotgun sequencing by GATC Biotech AG (Jakob-Stadler Platz 7, D-78467 Konstanz, Germany). The rearrangement point and the corresponding wild-type sequence were obtained by traditional sequencing, using an ABI Prism 3700 DNA Analyzer (Applied Biosystems). The sequence data have been submitted to the GenBank/EMBL/DDBJ database under the accession numbers: AY842844, AY842845, AY842846, AY842847, AY842848 and AY842849.
Design and isolation of the probes scattered on the *JBP1* chromosome

*X. major* Friedlin *JBP1* was localized at ∼18 kb downstream of one of the ends of chromosome 9 by a computer-based screen (BLAST search). Oligonucleotides were designed in order to make ∼500 bp (or ∼1 kb for probe 7) PCR fragments adjacent to selection marker or spaced by ∼500 bp or ∼1 kb, starting at ∼20 kb downstream of *JBP1* (going towards the distant telomere). PCR fragments were made using genomic DNA of *X. major* Friedlin as a template. Probe 1 was isolated by PCR using primers that were designed based on the sequence of a cosmid covering the *JBP1* locus and adjacent sequence of *L. tarentolae*. The PCR products were purified, labeled and hybridized on digested genomic DNA of *L. tarentolae*. The list of oligonucleotides used during this study is available upon request.

Quantitation of the copy number of the amplicon

The copy number of the amplicons in the various mutants was determined by quantitative autoradiography with a FLA-3000 apparatus (Fuji) using the Bas reader version 3.14 and Tina version 2.09 softwares. The signal obtained by hybridization on digested genomic DNA of *TarIIWT* was compared with the wild-type band in the KO *HYG* and KO *PUR* cell lines (Figure 1B, lanes 2 and 3). The inactivation of the second *JBP1* allele by transfection of the KO *PUR* construct into the KO *HYG* mutant failed, however, and resulted in a cell line containing two inactivated alleles, but maintaining a *JBP1* wild-type allele (Figure 1B, lane 4). All other attempts to obtain *JBP1* null mutants resulted in cell lines maintaining a wild-type allele. Failure to obtain a double gene inactivation suggests that *JBP1* is an essential gene in *Leishmania*, as also reported for the DHFR (dihydrofolate reductase) gene (1) and for the TR (trypanothione reductase) gene (3).

RESULTS

### *JBP1* null mutants cannot be generated in *L. tarentolae*

In order to inactivate *JBP1* in *Leishmania*, targeting constructs were made containing a selection marker (NEO, HYG or PUR) flanked by the 5′-UTR and 3′-UTR sequences of the *L. tarentolae* (Ltar) *JBP1* gene, and these were transfected into the *Leishmania TarIIWT* cell line. Integration of the cassettes into the *JBP1* locus was verified by hybridization, as schematically shown in Figure 1A. Transfection of the KO *HYG* and KO *PUR* constructs resulted in integration into the *JBP1* locus as shown by the appearance of new bands hybridizing with the *JBP1* 3′-UTR probe of, respectively, ∼3.4 and ∼7.0 kb in addition to the ∼4.7 kb wild-type band in the KO *HYG* and KO *PUR* cell lines (Figure 1B, lanes 2 and 3). The inactivation of the second *JBP1* allele by transfection of the KO *PUR* construct into the KO *HYG* mutant failed, however, and resulted in a cell line containing two inactivated alleles, but maintaining a *JBP1* wild-type allele (Figure 1B, lane 4). All other attempts to obtain *JBP1* null mutants resulted in cell lines maintaining a wild-type allele. Failure to obtain a double gene inactivation suggests that *JBP1* is an essential gene in *Leishmania*, as also reported for the DHFR (dihydrofolate reductase) gene (1) and for the TR (trypanothione reductase) gene (3). Our mapping data indicate that the wild-type allele in the *JBP1 KO* *HYG:KO PUR* mutant is present in a supernumerary *JBP1*-containing chromosome, since no DNA rearrangement was detected in the vicinity of the *JBP1* locus. Moreover, no ectopic insertion of *JBP1* was found into other chromosomes and the overall DNA content (ploidy) of the *JBP1 KO* *HYG:KO PUR* mutant was normal as determined by flow-cytometry (data not shown).

Targeting of the *JBP1* gene of *L. tarentolae* results in unexpected allelic ratios in some *JBP1* mutants

While trying to inactivate *JBP1*, we also generated mutants with a higher copy number of the inactivated alleles or the wild-type allele (compare the wild-type and inactivated alleles in lanes 1–4 versus lanes 5–8 in Figure 1B). Such unexpected allelic ratios were observed in the *JBP1 KO* *NEO*, KO

<table>
<thead>
<tr>
<th>Lanes</th>
<th>KO <em>PUR</em></th>
<th>KO <em>HYG</em></th>
<th>KO <em>NEO</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>++</td>
<td>+</td>
<td>++</td>
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<td>3</td>
<td>+</td>
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<td>4</td>
<td>+</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>8</td>
<td>-</td>
<td>+</td>
<td>+</td>
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</table>

Figure 1. Targeting of the *JBP1* locus and analysis of the mutant cell lines obtained. (A) Map of the *JBP1* locus before and after integration of the inactivation cassettes. Sequences flanking the *JBP1* locus were cloned upstream and downstream of selection markers. Sequences Y (polypyrimidine stretch) (30) and TUB (intergenic region of the alpha tubulin array of *Leishmania enriettii*) (49) were used for mRNA processing. The location of the NcoI sites and the 5′-UTR probe of, respectively, 3.4 kb and 7.0 kb are depicted. The drawing is approximately to scale. (B) Southern blot of genomic DNA digested with NcoI. The blot was hybridized with the *JBP1* 3′-UTR probe.
Presence of small linear amplicons in some JBP1 mutants

A chromosomal size fractionation by pulsed-field gradient electrophoresis, which allows the separation of all chromosome-sized DNA molecules of *Leishmania* (10,17,33,34), revealed the presence of new additional small linear chromosomes of ~100 kb only in the cell lines with an abnormal *JBP1* allelic ratio (Figure 2). Hybridization of a blot of the pulsed-field gel showed that the amplified markers were present in these small chromosomes (amplicons) (Table 1). The marker in the inactivation cassette used in the transfection that resulted in the appearance of the amplicons was always found in the amplicons, and in two out of four mutants there were two different amplified alleles, one with the marker inserted when the amplification occurred and the other already integrated in the genome prior to the transfection that induced the amplification.

**The JBP1 KO NEO amplicon consists of a long inverted repeat**

The fact that some amplicons contain two copies of the *JBP1* locus, each with a different marker or in one case with a marker gene and a wild-type *JBP1* allele (see Table 1), suggested that the amplicon contains a DNA duplication covering the *JBP1* locus. To verify this, the *JBP1 KO NEO* amplicon was extracted from a pulsed-field gradient gel as in Figure 2 and roughly mapped (data not shown). The sum of the size of all the bands arising from several digests fell short by almost half the size of the amplicon estimated by pulsed-field electrophoresis (~100 kb) (data not shown), indicating that the *JBP1 KO NEO* amplicon contained long sequences that were duplicated. The digested amplicon was hybridized with a telomeric probe, which recognized two bands in all the digestions tested, suggesting that the linear amplicons had telomeres at both ends (Figure 3A). A probe located ~13 kb upstream of the beginning of the *Ltar JBP1* locus (named probe 1, see Figure 4), (see Materials and Methods for a description of how probe 1 was isolated), recognized the same fragments that hybridized with the telomeric probe in some digests, indicating that the location of *JBP1* is subtelomeric (see for example the EcoRV digest in lane 2 in Figure 3A and B). These data also suggested that the two ends of the amplicon were located upstream of *JBP1*, implying that the *JBP1 KO NEO* amplicon had an inverted repeat structure. The differences in the size of the fragments recognized by the telomeric probe and probe 1 in the BamHI digest (lane 1 in Figure 3A and B) can be explained by the presence of restriction sites between the *JBP1* upstream probe (probe 1) and the telomeres, and by the presence of the KO NEO cassette inserted into the *JBP1* locus. The presence of two telomeric fragments is probably due to a difference in the number of telomeric repeats at the two ends of the amplicon, as the number of telomeric repeats is known to be highly variable in kinetoplastid parasites (35,36).

The inversion point in the *JBP1 KO NEO* amplicon corresponds to a repetitive sequence located downstream of the *JBP1* locus

In order to determine the inversion point of the long inverted repeat structure, we generated ~500 bp probes by PCR, scattered along the *JBP1*-containing chromosome of *L.major* Friedlin, the strain of reference for *Leishmania* sp., which has been completely sequenced (www.genedb.org) (see Figure 4 for the location of the different probes). *L.major JBP1* is located in chromosome 9, 18 kb upstream of the right end of the chromosome, consistent with the subtelomeric location of *JBP1* in *L.tarentolae*. The probes were used one by

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**Table 1. Genotype of the cell lines with a linear amplicon**

<table>
<thead>
<tr>
<th>Mutants</th>
<th>JBP1 KO NEO</th>
<th>JBP1 KO HYG:KO NEO</th>
<th>JBP1 KO PUR:KO HYG</th>
<th>JBP1 KO HYG:KO PUR:KO NEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker(s) in chromosome</td>
<td>JBP1, NEO</td>
<td>JBP1, HYG</td>
<td>JBP1, PUR</td>
<td>JBP1, PUR, NEO</td>
</tr>
<tr>
<td>Marker(s) in amplicon</td>
<td>JBP1, NEO</td>
<td>JBP1, HYG</td>
<td>PUR, HYG</td>
<td>HYG, NEO</td>
</tr>
</tbody>
</table>

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*Figures and diagrams are not provided in this text.*
one on digested genomic DNA of TarIIWT and JBP1 KO NEO. Hybridization of L.major Friedlin probes with genomic DNA of L.tarentolae is possible due to the high homology between the sequences of both species (~80–85% overall identity). Most probes hybridized to the same set of restriction fragments in the TarIIWT and JBP1 KO NEO, but more intensively with the DNA of the JBP1 KO NEO mutant (see Supplementary Data), implying that they are located in the amplicon, but not in the vicinity of the rearrangement point (Figure 4). Probes 10–13 hybridized with the same intensity with the DNA of TarIIWT and JBP1 KO NEO (see Supplementary Data), showing that they are located outside the rearrangement point (i.e. not in the amplicon) (Figure 4). However, a few probes located at respectively ~35 kb (probe 4) and ~41 kb (probes 7–9) downstream of the end of the JBP1 gene hybridized with a new band only present in the JBP1 KO NEO lane (see Supplementary Data), indicating that they are located at the rearrangement point of the amplicon (Figure 4). The extra bands hybridizing with both probe 4 and probe 9 in the JBP1 KO NEO mutant were cloned and sequenced. The corresponding sequences in TarIIWT were also cloned and sequenced in order to facilitate the identification of the rearrangement point (see Materials and Methods).

Comparison of the sequence of the rearrangement point of the JBP1 KO NEO amplicon with the corresponding sequences in TarIIWT revealed that the junction of both arms of the amplicon corresponded to a perfect repetitive sequence of ~222 bp, located three times in a direct or reverse (palindromic) orientation downstream of the JBP1 locus. We name these repeats RS1 to RS3 for repeated sequence (palindromic) orientation downstream of the JBP1 locus.

**Confirmation of the structure of the JBP1 KO NEO amplicon**

To verify that the amplicon has the structure summarized in Figure 6A, a PCR was done on genomic DNA of TarIIWT, JBP1 KO NEO and on the purified KO NEO amplicon using primers A and B oriented in the same direction as JBP1, and located at respectively ~410 bp upstream of the RS2 repeat and ~556 bp downstream of the RS3 repeat (Figure 6A). These two primers should be spaced by ~1200 bp (counting the ~222 bp RS repeat) on each side of the rearrangement point of the KO NEO amplicon if it has the structure presented in the Figure 6A. A PCR product having the expected size was obtained only using DNA from the JBP1 KO NEO cell line and the purified amplicon (alone or mixed with TarIIWT genomic DNA) (Figure 6B, left panel, lanes 3, 5 and 6). The PCR product was sequenced and was found to correspond to the predicted rearrangement point. No PCR product was obtained using a JBP1 KO NEO revertant cell line that lost the amplicon by culturing in the absence of drug selection (Figure 6B, left panel, lane 4). No PCR product was obtained either on the

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**Figure 3.** Analysis of the telomeric ends of the JBP1 KO NEO amplicon. Southern blot on digested JBP1 KO NEO amplicon isolated from the pulsed-field gel. The blots were hybridized with a telomeric probe (A) and with probe 1 (see Figure 4), located ~13 kb upstream of the JBP1 locus (B). Lane 1, BamHI; and lane 2, EcoRV.

**Figure 4.** Schematic representation of the results of the hybridization experiments done to detect the rearrangement point in the JBP1 KO NEO amplicon. The small rectangles represent the approximate location in the JBP1 chromosome (Chr 9) of the different probes that were generated by PCR on genomic DNA of L.major Friedlin. The white rectangles correspond to the probes that are not present in the amplicon, the black rectangles depict the probes that are present in the amplicon. The gray rectangles correspond to the probes that detect the rearrangement point. The bars indicate the approximate locations of the EcoRV sites around the JBP1 locus in L.tarentolae. The small arrows indicate the location of the RS1, RS2 and RS3 repeats. Their orientation is indicated by the direction of the arrow. The triangles represent the telomeric repeats. The JBP1 gene is depicted by the yellow rectangle. The drawing is approximately to scale.
purified amplicon using primers C and D located on both sides of RS3 showing that at least one of these primers (primer D) is located outside the rearrangement point (Figure 6B, right panel, lane 5). Note that a PCR product is obtained with primers C and D on the genomic DNA of the JBP1 KO NEO mutant since this cell line still has a wild-type chromosome (Figure 6B, right panel, lane 3).

**Structure of the JBP1 KO NEO HYG and JBP1 KO PUR:KO HYG amplicons**

The experiments done with the JBP1 KO NEO amplicon were repeated for the JBP1 KO HYG:KO NEO and JBP1 KO PUR:KO HYG cell lines and showed that these amplicons have a similar inverted repeat structure (data not shown). The inversion points in the JBP1 KO HYG:KO NEO and JBP1 KO PUR:KO HYG amplicons correspond to the same repeated sequence found at the junction of the KO NEO amplicon. However, in the former amplicons, the rearrangement happens to involve the RS1 and the RS2 repeats, also oriented in a reverse (palindromic) fashion, rather than RS2 and RS3 (see Supplementary Data). It should be noted that the length of the homology between the RS1 and RS2 repeat is ~210 bp instead of ~222 bp. Although we did not map the JBP1 KO HYG:KO PUR:KO NEO amplicon, we suspect that its rearrangement involved another repeat, called RS4, present more downstream of JBP1 than RS3 in *L. major* Freidlin (see also Discussion). This would explain its longer size compared to the other amplicons as shown by pulsed-field gradient electrophoresis (compare lane 8 versus lanes 5–7 in Figure 2).

The amplicons cannot be generated by drug pressure on a cell line having one marker integrated in the JBP1 locus

Generation of a linear DNA amplicon following a targeting event has not been described in *Leishmania* before, or in any other organism. To determine whether the amplification was caused by the transfection itself or was due to a highly recombinogenic property of the JBP1 locus, we tried to generate amplicons by increasing the drug pressure on the JBP1 KO HYG mutant, which has one copy of the HYG marker integrated in the *JBP1* wild-type chromosome. In *Leishmania*, this procedure often results in DNA amplification of genes involved in the resistance to the drug used (5,16).
increased the hygromycin B concentration in which \textit{JBP1 KO HYG} was cultured stepwise by a factor two until it reached 2 mg/ml (40 times the normal concentration at which \textit{L.tarentolae} is cultured). After culture for over three months at this concentration, we found no amplicons by hybridization on digested genomic DNA and pulsed-field gel electrophoresis (data not shown), suggesting that the integration of the inactivation cassette facilitated the formation of the amplicon.

**DISCUSSION**

In our attempts to disrupt both alleles of \textit{JBP1}, an essential gene in \textit{Leishmania}, we obtained mutants that appear to have a supernumerary \textit{JBP1}-containing chromosome, as exemplified by the \textit{JBP1 KO HYG:KO PUR} mutant and mutants containing linear plasmids (amplicons) with a long inverted duplication (Figure 6A). Whereas mutants with supernumerary chromosomes have been generated before in \textit{Leishmania} by attempts to knock out essential genes (1–3), the linear plasmids are new and their unusual structure provides detailed information about the DNA rearrangement that generated them.

\textit{Leishmania} is known to readily amplify DNA segments if challenged, be it by increasing drug pressure (5,10,16,37), by attempts to target essential genes (1–3), by nutrient stress or by subcloning (38). Amplification is even frequently seen in unselected laboratory stock (15). Linear DNA amplicons with an inverted repeat structure similar to the one described here have been described before in \textit{Leishmania} in response to drug selection (39) and in the absence of selection (40,41). The generation of circular DNA amplicons by the circularization of the \(\alpha\)-tubulin locus has also been reported following attempts to inactivate this essential gene in \textit{Leishmania} (42). There are no previous reports, however, of linear amplicons with an inverted repeat structure, having different markers in the two arms generated following a targeting event. We attribute the rarity of this type of amplicon to four factors (see Figure 7): (i) the targeting of an essential gene; (ii) the subtelomeric location of \textit{JBP1}, which may facilitate formation of stable linear amplicons; (iii) the inferred presence of a DNA replication origin upstream of \textit{JBP1}; and (iv) the presence of inverted repeats not far downstream of \textit{JBP1} facilitating strand switch in the replication fork.

**Model for linear amplicon formation**

Figure 7 presents our preferred model for the formation of the amplicons. In this model, the inactivation cassette integrates into one arm of a replication fork that recently passed through the \textit{JBP1} locus. Insertion of the cassette might cause the replication fork to stall [see Michel et al. (43)]. Reversal of the fork would allow the RS3 repeat of the leading strand to anneal to the single-stranded RS2 repeat of the lagging strand (Figure 7B). This template strand switch is necessary to explain the presence of two different markers at the two ends of most amplicons. Peeling of the amplicon would allow the parental strands to snap back together, giving rise to a partial heteroduplex that would need to be repaired, for instance by gene conversion (Figure 7E). If the choice of
the strand to be repaired is random, half the targeting events will result in a new marker in the targeted chromosome. The other half will leave the original gene in place. This is what we find as shown in Table 1. The alleles that get amplified and end up in the linear plasmids are the one in the inactivation cassette and the one that was targeted by the cassette. Similar models involving a template switch in a DNA replication fork have been proposed to explain the formation of amplicons bearing an inverted duplication in mammalian cell lines (44–46).

Our model is based on the following assumptions. The fact that the two arms of the amplicons contain different marker genes (or a wild-type gene and a marker) suggests that the targeting construct must have hit replicating DNA (see Figure 7). Hence, we infer the presence of an origin of replication located upstream of JBP1. The presence of a short inverted repeat at the inversion point of the linear plasmid indicates that the long inverted repeat structure of the plasmid arose probably from a template switch of the leading strand in a replication fork, as depicted in Figure 7. We propose that the integration of the inactivation constructs affected the progression of the replication fork allowing the DNA strand switch at the origin of the amplicon formation, but this is speculative.

It is obvious that the time window available for the generation of these plasmids must be narrow: targeting must take place after the replication fork has passed the JBP1 locus. One of these copies is targeted by the inactivation construct, which replaces JBP1 by NEO (A). The perturbation caused by the targeting could cause a stalling of the replication fork. Reversal of the fork would enable the RS3 repeat of the leading strand to anneal with the not yet replicated RS2 repeat of the lagging strand (B). Replication would restart using the other strand as a template followed by the synthesis of the complementary strand (C). Peeling of the misreplicated section would lead to an amplicon having an inverted repeat structure and two single-stranded DNA having on one strand JBP1 and on the other the NEO marker (D). Annealing of the strands would create a heteroduplex that would be repaired (E). The newly introduced marker would end up in the JBP1 chromosome only in half of the events, depending on which strand is repaired (F). The leading strand of the replication fork is shown in orange, the lagging strand in blue. The RS and telomeric repeats, as well as the JBP1 and NEO genes, are depicted as in Figure 6.

Figure 7. Postulated model to explain the generation of the JBP1KO NEO amplicon. We propose that replication starts upstream of JBP1. Two copies of JBP1 are generated after the replication fork has passed the JBP1 locus. One of these copies is targeted by the inactivation construct, which replaces JBP1 by NEO (A). The perturbation caused by the targeting could cause a stalling of the replication fork. Reversal of the fork would enable the RS3 repeat of the leading strand to anneal with the not yet replicated RS2 repeat of the lagging strand (B). Replication would restart using the other strand as a template followed by the synthesis of the complementary strand (C). Peeling of the misreplicated section would lead to an amplicon having an inverted repeat structure and two single-stranded DNA having on one strand JBP1 and on the other the NEO marker (D). Annealing of the strands would create a heteroduplex that would be repaired (E). The newly introduced marker would end up in the JBP1 chromosome only in half of the events, depending on which strand is repaired (F). The leading strand of the replication fork is shown in orange, the lagging strand in blue. The RS and telomeric repeats, as well as the JBP1 and NEO genes, are depicted as in Figure 6.
Other models of DNA amplification

Besides our ‘template-switch in a replication fork’ model shown in Figure 7, three other possibilities can be considered that could explain the generation of plasmids. Reversal of the replication fork might generate single newly synthesized strands, which can partially snap back on themselves by means of the RS repeats. If this u-turn is followed by self-copying and replication of the duplex formed, a plasmid with the same marker on both arms will arise, as exemplified by the JBP1 KO HYG:KO NEO plasmid (which cannot be explained by our strand-switch model) (see Table 1). This mutant has NEO as the only marker present in the amplicon and might have been formed by an auto-annealing of the branch of the replication fork bearing the NEO marker. Formation of amplicons by auto-annealing of palindromic repeats followed by self-copying of one DNA strand is a preferred model to explain the formation of amplicons in Leishmania (5,9,17).

A second possibility to generate the amplicons is to cut off the parental DNA at the replication fork, as suggested by Nalbantoglu and Meuth (44). What argues against this alternative is that the replicating chromosome from which the plasmid arises remains intact (data not shown). The broken chromosome could repair itself, however, by copying homologous DNA [see Michel et al. (43)]. Since the intact chromosome contains either of the markers present in the amplicon and not the marker present in the other chromosome, this repair mechanism remains implausible, as it would have to involve the newly made plasmid.

A third popular model of DNA amplification involves the over-replication of a portion of the genome causing an ‘onion-like’ structure, followed by the resolution or recombination of the over-replicated segment (16,18,46). This model cannot easily explain our observation, however, that the inactivation construct that caused amplification is always found in the amplicon and not in the original chromosome (Table 1). The ‘onion-skin’ model would rather predict that this marker is always present in the amplicon and not in the original chromosome, in the case where the construct would integrate in the over-replicated sequence, or that it is found in both the amplicon and the JBP1 chromosome, if the cassette integrated prior to over-replication and formation of the amplicon. It is nevertheless possible that both events occurred in our case, but we find that unlikely.

Amplicon copy number

In all mutants analyzed, the amplicons are present in multiple copies, even though one would expect that one copy of the marker replacing the JBP1 gene should be enough to confer resistance to the drugs at the concentration used. Several factors probably contribute to this plasmid multiplicity: one factor is the use of a NEO gene in our experiments that contains a mutation (see Materials and Methods), which gives a diminished activity towards genetin (G418). This results in a marginal resistance if a single NEO copy is present per cell (data not shown). In the generation of the JBP1 KO NEO mutant, which has also one NEO copy in the JBP1 chromosome, we obviously used a concentration of G418 that was too high for the cells to survive without gene amplification. The mutant NEO gene confers high-level resistance to paromomycin, however, and when the JBP1 KO NEO cells were transferred to paromomycin they lost the plasmid. The multiplicity of linear plasmids can also be explained by the fact that our plasmids do not contain centromeres and are rapidly lost in the absence of selection, like other linear plasmids in Leishmania. Since cells without a single copy of the plasmid will die in the presence of selection (since most amplicons contain markers that are not present in the wild-type chromosome, see Table 1), there must be a strong selection in the mutant population for clones that can transmit at least one plasmid copy to each daughter cell. This must drive up the copy number and we think that this partially explains why our mutants contain on average ~4–5 plasmid copies per cell. If plasmid segregation is random, a cell with 4 copies (before replication) will generate less than 1% of daughter cells without a single plasmid copy. Such a low loss should not measurably affect the growth of the population.

Implication of repetitive sequences in the generation of the linear amplicons

At the inversion point of the three amplicons that we mapped, we found repeats that we hold responsible for the template switch in a stalled replication fork. The tendency of autoannealing of repeats RS1-3 is limited as we were unable to generate amplicons after increasing the drug pressure, a process that often yields amplicons in Leishmania (5,16). This suggests that the integration of the targeting cassette facilitates the template switch at the origin of the amplicon formation, e.g. by stalling the replication fork. Repeats (direct or inverted) are often associated with DNA amplification in Leishmania. For instance, repeats were proposed to be responsible for the formation of circular and linear amplifications of the H locus arising from arsenite or methotrexate selection (9,10). Interestingly, a repetitive DNA sequence having a moderate homology (~50%) with the sequence found at the rearrangement point of our amplicons (RS repeats) is also present at the same location in the chromosome 9 of L.major Friedlin where the JBP1 gene is located. A computer-based genome screen of the repetitive element found in chromosome 9 of L.major Friedlin revealed that this sequence is found in many other chromosomes (data not shown). Moxon et al. (48) have proposed that ‘mutation rates vary among sites in the genome and that this variation is adaptive, because it promotes evolutionary flexibility in the face of environmental change, without necessarily increasing the overall load of deleterious mutations’. These highly mutable loci are called contingency genes, because they ‘facilitate the efficient exploration of phenotypic solutions to unpredictable aspects of the host environment’ (48). Leishmania can respond to some environmental challenges with a specific type of mutation, i.e. gene amplification, and the repeats associated with this amplification fulfill a clear contingency function (9,16). Possibly, the RS repeats studied here fulfill a similar role. It could be worth looking at other already described amplicons in Leishmania to see whether the formation of some of them could be explained by these RS repeats. The contingency genes that might be amplified through these RS repeats remain to be identified.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.
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