The deduced amino acid sequence of the region downstream of the reverse transcriptase (RT) motif of the Trypanosoma cruzi L1Tc non-LTR retrotransposon shows a significant homology with the sequence coding for proteins with RNase H activity from different organisms and retroelements. The 25-kDa His$_6$-tagged recombinant protein bearing only the L1Tc RNase H domain, named RHL1Tc, exhibits RNase H activity as measured on the $[^{3}H]$poly(rA)/poly(dT) hybrid used as substrate as well as on specific homologous and heterologous [$^{32}$P]RNA/DNA hybrises. The mutation of the conserved aspartic acid at position 39 of the enzyme catalytic site, but not of the serine at position 56 (non-conservative amino acid), abolishes protein RNase H activity. The RNase H activity of the RHL1Tc protein is Mg$^{2+}$-dependent, and it is also active in the presence of the Mn$^{2+}$ ion. The optimal condition of RNase H activity is found at pH 8 and 37 °C, although it also has significant enzymatic activity at 19 °C and pH 6. However, it cannot be excluded that the RNase H activity level and its optimal conditions may be different from that of a protein containing both RT and RNase H domains.

Sequencing of the human genome and those of several other organisms has revealed the existence of a high number of retrotransposable elements such as LINEs (long interspersed nucleotide elements) and SINEs (short interspersed nucleotide elements) (1). A common characteristic of these elements is that they are mobilized into the genome via an intermediate RNA. LINEs are considered autonomous elements because they encode the proteins involved in their own transposition process. In contrast, because SINEs, such as Alu sequences, lack a protein-coding capacity it has been suggested that they use the enzymatic machinery of LINEs in trans (1). In the human genome there exist from 3000 to 5000 full-length copies of LINE-1 dispersed throughout the genome, accounting for 15–17% of its mass (2). Recent studies have involved these elements in relevant biological processes such as gene regulation (3, 4), modeling of the genome shape (5), or DNA-shuffling phenomena (6). Moreover, it has been shown that insertion of LINE in certain genes affects the outcome of some diseases and has been associated with the generation and progression of certain cancers (7, 8).

LINEs use a transposition mechanism in which the reverse transcription of the RNA template is primed by the release of a 3’-hydroxyl group (9) after cleaving chromosomal DNA with an endonuclease activity encoded by the element (10, 11). To synthesize the second strand DNA, the RNA template has to be removed from the RNA/cDNA hybrid by an RNase H activity, which may be supplied by the host cell (12). Recent studies (13) have described in some non-LTR retrotransposons the existence of sequences with a certain homology to proteins with RNase H activity. However, RNase H activity has still not been demonstrated to be present in the non-LTR retrotransposons.

L1Tc is a highly repetitive non-LTR retrotransposon widely distributed in the genome of the parasite Trypanosoma cruzi (14, 15), which is actively transcribed into poly(A)$^+$ RNA. The first L1Tc-characterized copy corresponds to a cDNA containing three ORF (14). The second and third ORF showed significant homology with the RT and cysteine motifs of the pol and gag genes, respectively, from retrovirus and LTR retrotransposons. The first ORF exhibited an endonuclease domain, and the recombinant protein encoded by this ORF showed apurinic/apyrimidine (AP) endonuclease activity (16) and 3’-phosphatase and 3’-phosphodiesterase activities (17). Studies of the genomic organization and distribution of L1Tc in T. cruzi show the existence of some L1Tc copies formed by a single ORF of 5 kb that contains the endonuclease, RT, and cysteine motifs (15). Interestingly, analysis of the sequence located downstream of the RT motif reveals a significant homology with the RNase H domain present in retroviruses and Escherichia coli. In the present study we have shown that the T. cruzi L1Tc recombinant protein encoded by this domain has RNase H activity as measured on the $[^{3}H]$poly(rA)/poly(dT) hybrid as well as other homologous and heterologous RNA/DNA hybrid substrates. We have also shown the ion requirements, the temperature, and the optimal pH conditions for RNase H activity of the recombinant protein encoded by L1Tc.

**EXPERIMENTAL PROCEDURES**

Molecular Cloning of the L1Tc RNase H Domain—Two oligonucleotides, RH5’ (5’-TGTGGAGCTCCCGATAGACC-3’) and RH3’ (5’-
TACTACTGGACTTGGTGAGCTGGGG-G3') were used to amplify by PCR the homologous LiTc region, which in the LiTc cDNA copy (14) is between positions 3571 and 4182, using the pBAC14 vector (15) as template. SacI and SalI sites (underlined) were generated ad hoc in the amplified fragment, which was subsequently digested with those enzymes and cloned into the pQEC expression vector (Qiagen). The resulting plasmid, containing 603 bp of the amplified region, was called pQBRH1tC (GenBank™AY045718). Two copies of the amplified fragment were in-frame cloned into the pQE30 expression vector, generating the vector pQRH2L1tC.

Site-directed Mutagenesis of the LiTc RNase H Domain—Mutagenesis of the LiTc RNase H domain was performed using a PCR-based technique (described in 18), with pTH9252 as template and respective primers. The amplified fragments were used to replace the coding region of the RNase H domain was cloned into the pQE30 expression vector, generating the vector pQRH293NLtC and pQRH566ALtC, respectively. The introduced mutations were confirmed by DNA sequencing.

Expression and Purification of Recombinant Proteins.—The E. coli M15 strain was transformed with the constructs pQBRH1tC, pQRH293NLtC, and pQRH566ALtC and the E. coli TOP3 strain with pQRH2L1tC vector. The recombinant proteins were overexpressed in cultures grown at OD600 = 0.6 with 1 mM IPTG for 3 h at 37 °C, were purified by nickel affinity chromatography. For this purpose, cell pellets were resuspended in buffer containing 50 mM sodium phosphate and 300 mM sodium chloride, pH 7.4, incubated with 1 mg/ml lysozyme for 15 min, sonicated for 5 min, and centrifuged for 10 min at 12,000 × g for 4 °C. Soluble fractions were incubated with NTA-agarose resin (Qiagen) for 1 h at room temperature. Subsequently, the resins were washed three times with 20 ml of buffer containing 50 mM sodium phosphate, 300 mM sodium chloride, 0.1% Nonidet P-40, 0.2% sodium azide, and 20% glycerol. Fractions containing the recombinant proteins were eluted with 50 mM imidazole and repurified to homogeneity. Protein concentration was determined by the Bradford method (19).

Enzymes.—The following enzymes were used: E. coli RNase H (Roche; HIV-1 RT protein containing the RNase H domain (provided by Drs. A. Berzal-Herranz and A. Barroso-delJesus). 2 µg of the amplified DNA were used in vitro transcription using T7 RNA polymerase promoter sequence (underlined). Plasmid pASW was used as template. pASW is a derivative of the pUC19 plasmid, which contains the 14-nucleotide-long wild type target of the (−)dNTRSV (negative polarity satellite RNA of the tobacco ring spot virus) hairpin ribozyme cloned into the coding region of the enzyme β-galactosidase (provided by Drs. A. Berger-Herranz and A. Barroso-delJesus). 2 µg of the amplified DNA were used for in vitro transcription using T7 RNA polymerase (see above). The 414-nt-long RNA was radiolabeled and purified, and the specific activity was measured as described for LiTc-(1203-1352) [32P]RNA. The p1 oligonucleotide 5′-TGAATCAACAGACTGTTCA-GAGGTCTGGTCACCCGGG-G3′, complementary to nucleotides 119–158 in the β-galactosidase [32P]RNA, was hybridized to radiolabeled RNA as described above except that 0.25 pmol of β-galactosidase [32P]RNA and 5 pmol of p1 oligonucleotide were used.

RNase H Activity Assays.—The nonspecific [32P]RNA hybrid assay, using the (HiPolyra/PolydT) hybrid as substrate, was performed essentially as described (20) except that 1 µCi/ml hybrid in a final volume of 25 µl was used. The master buffer reaction (25 µl-Tris, pH 8, 5.0 mM magnesium chloride, 90 mM sodium chloride, 1.5% dextran blue) was incubated at 37 °C for 2 min, and stopped with the addition of 2× stop solution (20% formamide, 17 mM EDTA, 0.025% xylene cyanol, 0.025% bromphenol blue) (v/v). Finally, samples were denatured at 95 °C for 2 min, cooled on ice for 5 min, and electrophoresed through a denaturing 6% polyacrylamide, 8 M urea gel. The gel was wrapped in plastic and exposed to Kodak X-omat autoradiographic film.

RESULTS

Presence of an RNase H Domain in LiTc Retrotransposon of T. cruzi—Sequence analysis of different genomic LiTc copies from a T. cruzi library constructed in pBAC vector showed that in some clones the LiTc element is formed by a single open reading frame of 5 kb (15). Remarkably, a sequence with significant amino acid homology to enzymes with RNase H activity from E. coli and HIV-1 was found (Fig. 1B) in these copies downstream of the reverse transcriptase (RT) domain (Fig. 1A). Thus, a 139-amino acid region present in the LiTc copy, which was contained in the pBAC14 clone (15), showed 26.9 and 31.6% identity with the RNase H coding sequence from HIV-1 and E. coli (Z-score of 8 and 6, respectively). Moreover, the amino acids forming the active site (essential for catalysis of the E. coli RNase H enzyme) as well as the neighboring region of the catalytic site (21, 22) were conserved in the LiTc protein encoded by LiTc (Fig. 1B).

To determine whether the LiTc region containing the putative RNase H domain codes for a protein with RNase H activity, a 603-bp DNA fragment from pBAC14 containing the LiTc RNase H domain was cloned into the SacI and SalI sites of the pQE30 vector. In addition, two copies of the amplified fragment were also cloned in-frame into the pQE30 vector. The resulting vectors, pQBRH1tC and pQRH2L1tC, respectively, were transformed into E. coli M15 and TOP3 strains, and the recombinant proteins were overexpressed after IPTG induction. Two intensely stained bands of −25 and 40 kDa corresponded, respectively, to the expected sizes of the RHL1tC and RH2L1tC by heating at 95 °C for 2 min followed by two incubations for 15 min on ice and at 37 °C, respectively.

The β-galactosidase α-peptide (β-gal) [32P]RNA/dDNA hybrid was used as heterologous substrate. The DNA template for RNA synthesis was obtained by PCR using p1 (5′-TTATCCGTGGCGGTGTAGACG-3′) and p5 (5′-TATGCTATCCTGCTGATT-3′) oligonucleotides. Primer C1 contains in 5′ the T7 RNA polymerase promoter sequence (underlined). Plasmid pASW was used as template. pASW is a derivative of the pUC19 plasmid, which contains the 14-nucleotide-long wild type target of the (−)dNTRSV (negative polarity satellite RNA of the tobacco ring spot virus) hairpin ribozyme cloned into the coding region of the enzyme β-galactosidase (provided by Drs. A. Berger-Herranz and A. Barroso-delJesus). 2 µg of the amplified DNA were used for in vitro transcription using T7 RNA polymerase (see above). The 414-nt-long RNA was radiolabeled and purified, and the specific activity was measured as described for LiTc-(1203-1352) [32P]RNA. The p1 oligonucleotide 5′-TGAATCAACAGACTGTTCA-GAGGTCTGGTCACCCGGG-G3′, complementary to nucleotides 119–158 in the β-galactosidase [32P]RNA, was hybridized to radiolabeled RNA as described above except that 0.25 pmol of β-galactosidase [32P]RNA and 5 pmol of p1 oligonucleotide were used.
The enzymatic activity of the protein encoded by the L1Tc RNase H domain was also analyzed by monitoring its cleavage efficiency on homologous and heterologous uniformly labeled RNA transcripts hybridized to an internal complementary oligonucleotide. Two different labeled RNA/DNA hybrids were used. One corresponded to the coding region of \( \beta \)-galactosidase, which does not contain significant sequence homology with L1Tc retrotransposon. The other one corresponded to an internal region of L1Tc. Thus, a 414-nt RNA fragment of \( \beta \)-galactosidase was annealed to a complementary 39-nt oligodeoxyribose (Fig. 4A), and incubated with RHL1Tc, RH39NL1Tc, RH56AL1Tc, RH2L1Tc, and the E. coli and HIV-1 RNase H proteins. The purified recombinant protein encoded by the L1Tc RT domain (RTL1Tc) and a reaction without enzyme were used as negative controls. Analysis of the DNA cleavage products on a denaturing 6% polyacrylamide, 8 M urea gel showed that the RHL1Tc, RH56AL1Tc, and RH2L1Tc proteins cut the labeled RNA strand of the RNA/DNA hybrid releasing, in a similar form to the proteins from E. coli and HIV-1, two major RNA fragments of \(-120 \) and \(260\) nucleotides, which correspond to the oligonucleotide non-annealed ends of the RNA strand (Fig. 4A, lanes 4, 5, and 8). However, as occurs with nonspecific substrates, the RH39NL1Tc mutant protein did not show enzymatic activity.
RNase H Activity in Trypanosoma cruzi L1Tc LINE

(FIG. 4A, lane 9). Slight differences were observed in the size of the fragments generated by each enzyme because of heterogeneity in the cleavage sites of RNase H proteins assayed. The RHL1Tc and RH2L1Tc proteins showed the same cleavage pattern, although RH2L1Tc hydrolyzed the substrate more efficiently than RHL1Tc. As was expected, no RNase H activity was detected in the reaction containing the RT protein encoded by L1Tc RNA/DNA hybrid. Like the L1Tc-(1203–1352) RNA/DNA hybrid, the RH2L1Tc protein showed two major fragments (FIG. 4B, lane 9). Moreover, the RH2L1Tc protein also cut the L1Tc-(1203–1352) RNA/DNA hybrid substrate more efficiently than the RHL1Tc protein did. The E. coli RNase H protein efficiently cleaved the hybrid, releasing two major fragments (FIG. 4B, lane 1) similar to those released by the RNase H encoded by L1Tc. However, the cleavage pattern of the HIV-1 RT protein from HIV-1 was different (FIG. 4B, lane 2), probably because of particular structure or sequence determinants present in the L1Tc-(1203–1352) RNA/DNA hybrid.

In order to test RNase H activity with a homologous substrate, an L1Tc RNA/DNA hybrid molecule was formed between a 227-nt-long transcript derived from L1Tc (see “Experimental Procedures”) and a 21-nt-long complementary oligonucleotide. The uniformly labeled RNA fragment/DNA hybrid was incubated with different RNase H proteins (FIG. 4B). Analysis of the RNA cleavage products generated by the RHL1Tc, RH5S6AL1Tc, and RH2L1Tc proteins showed two major fragments of ~110 and 95 nt corresponding to the non-annealed ends of the RNA strand (FIG. 4B, lanes 4, 5, and 8). Like the β-galactosidase hybrid, the RHD09NL1Tc mutant protein did not show RNase H activity on the L1Tc-homologous substrate (FIG. 4B, lane 9). Moreover, the RH2L1Tc protein also cut the L1Tc-(1203–1352) RNA/DNA hybrid substrate more efficiently than the RHL1Tc protein did. The E. coli RNase H protein efficiently cleaved the hybrid, releasing two major fragments (FIG. 4B, lane 1) similar to those released by the RNase H encoded by L1Tc. However, the cleavage pattern of the HIV-1 RT protein from HIV-1 was different (FIG. 4B, lane 2), probably because of particular structure or sequence determinants present in the L1Tc-(1203–1352) RNA/DNA hybrid.

Enzymatic Requirements of RHL1Tc—To determine the ion requirements of the RHL1Tc enzyme, RNase H activity was analyzed at different Mg²⁺ and Mn²⁺ ion concentrations at pH 8. The RHL1Tc protein was determined to require Mg²⁺ or Mn²⁺ with a [³²P]poly(rA)/poly(dT) hybrid substrate with an optimal concentration of 5 mM Mg²⁺ or 1 mM Mn²⁺ (FIG. 5, A and B). The RNase H activity level in the presence of 5 mM Mg²⁺ and 1 mM Mn²⁺ ions. Data represent the released cpm of three independent assays after subtraction of the background values.

FIG. 4. Cleavage pattern on labeled RNA/DNA hybrids. Activity on the [³²P]RNA/DNA hybrid substrates corresponding to the RNA of β-galactosidase (A) and L1Tc-(1203–1352) (B). Schematic representations of the employed substrates and the expected products in the RNase H assays are indicated at the left. The reactions were carried out as described under “Experimental Procedures.” The products were electrophoresed through a 6% polyacrylamide gel. Major cleavage products were detected in the reaction containing the RT protein encoded by L1Tc, RH2L1Tc, RH5S6AL1Tc, and RHD39NL1Tc recombinants proteins; lane 5, 0.62 pmol of RH2L1Tc protein; and lanes 6 and 7, reaction without enzyme. 1/10 volume of the reaction was loaded when E. coli and HIV-1 enzymes were used (panels A and B, lanes 1 and 2).

FIG. 5. Requirements for Mg²⁺ and Mn²⁺. RNase H activities from E. coli (□), HIV-1 (○), and L1Tc (■) in the presence of 1, 2.5, 5, and 7.5 mM of Mg²⁺ ion (panel A) and 0.5, 1, and 2 mM Mn²⁺ ion (panel B). The activity was measured as described under “Experimental Procedures” and is represented as percentage of activity relative to the maximal value obtained for each enzyme. Data represent the average of three independent experiments, once the background values were subtracted. C, comparison of RHL1Tc maximal activity in the presence of 5 mM Mg²⁺ and 1 mM Mn²⁺ ions. Data represent the released cpm of three independent assays after subtraction of the background values.
The RNase H protein encoded by the L1Tc element is Mg$^{2+}$ dependent; it is also active in the presence of the Mn$^{2+}$ ion. The optimal pH and temperature conditions are pH 8 and 37 °C. These conditions are similar to those described for the E. coli RNase H and HIV-1 RT enzymes. However, it cannot be excluded that enzyme optimal conditions could be different for a protein containing both RT and RNase H domains, as has been described in retrovirus (29, 33). Remarkably, the RHL1Tc protein is more temperature and pH permissive than the HIV-1 protein. The isolated HIV-1 RNase H domain increases activity when a polyhistidine tag is added (28, 29). Although the basic amino acid content in the putative handle region of the isolated L1Tc RNase H domain is double that present in the HIV-1 RNase H protein, we cannot exclude the contribution by the hexahistidine tag to the detected enzymatic activity, which the RHL1Tc recombinant protein carries at its N-terminal end.

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Sequence analysis has evidenced that a 603-bp-long region from a L1Tc genomic copy has a significant degree of sequence similarity with different RNase H proteins and is conserved in similar positions to the amino acids forming the active site described for the RNase H enzyme family (22). This sequence has been cloned into an expression vector, overexpressed, and purified to homogeneity. In this study, we report that the purified RHL1Tc recombinant protein containing the L1Tc RNase H domain has RNase H activity on various RNA/DNA hybrid substrates. Remarkably, the RH39NL1Tc mutated protein, which presents a substitution at the conserved aspartic acid residue at position 39, is devoid of cleavage activity. This result indicates this residue is also essential for enzymatic activity of the L1Tc-encoded enzyme, as has been described for the E. coli, human, and retrovirus RNase H proteins (26). On the other hand, as expected, the RHS6AL1Tc mutated protein, bearing a substitution at the non-conserved serine amino acid at position 56, is as active as the RHL1Tc wild type recombinant protein.

Enzymatic activity detected for the RHL1Tc protein was similar to that described for some retroelements, such as the endogenous HERV-K retrovirus (27), and significantly lower than that observed for the E. coli RNase H and HIV-1 RT enzymes. It is worth mentioning that other isolated RNase H domains from retroviruses have been shown to present a lower enzymatic activity than the protein containing both RT and RNase H domains (28, 29). On the other hand, in HIV-1 (30) but not in MuLV (31), the RNase H domain is not an active protein by itself. It has been suggested that the presence of a high content of basic residues in some regions of the protein could be necessary for activity. The MuLV protein contains 23% basic amino acids in the α-C, -B, and -D regions, the handle region, whereas the HIV-1 protein only has 3.6% (23, 29, 31). It has been suggested that these basic amino acids function in substrate affinity and RNase H stability (32). The isolated HIV-1 RNase H domain increases activity when a polyhistidine tag is added (28, 29). Although the basic amino acid content in the putative handle region of the isolated L1Tc RNase H domain is double that present in the HIV-1 RNase H protein, we cannot exclude the contribution by the hexahistidine tag to the detected enzymatic activity, which the RHL1Tc recombinant protein carries at its N-terminal end.

RNase H enzymes are widely distributed among prokaryotes and eukaryotes (24), and it is believed that they participate in DNA replication processes by removing the upstream RNA primers of Okazaki fragments (25). During the retrotransposition event of retrovirus and retrovirus-like elements an RNase H activity encoded by its own elements removes the RNA template of the generated RNA/cDNA hybrid in order to allow second strand DNA synthesis. It has been suggested that in the case of LINE the RNase H activity needed for completing the retrotransposition process could be provided by the host cell (12). A putative RNase H domain has recently been reported (13) in some non-LTR retrotransposons, in an analogous position to that described for retroviruses and LTR-retrotransposons. However, it has not yet been demonstrated that the putative RNase H domain is endowed with enzymatic activity.
RNase H enzyme from HIV-1. This divergent pattern obtained with the HIV-1 RT protein may be due to the presence, in the hybridized LITc RNA region, of a 5-nt-long purine stretch that could be acting as a polypurine tract (PPT)-like sequence. It has been reported that purine-rich sequences can act as PPT-like sequences for the RNase H enzyme from HIV-1 containing the RT domain (35).

Remarkably, it has been observed that the recombinant protein RH2L1Tc containing two in-frame copies of the RNase H domain cleaves the substrates more efficiently than the RHL1Tc protein. The higher activity of the dimeric protein could be caused by a special or more active protein conformation, or perhaps the enzyme binds the substrate as a dimer as has been reported for the MuLV enzyme (36). Further studies will be necessary for clarification of the role that the conformational structure of the LITc RNase protein plays in RNase H activity. Our discovery of RNase H enzymatic activity associated with the LITc RNase H domain should contribute to understanding the LINE retrotransposition mechanism and should reinforce the idea of the autonomy of such retroelements as they encode the enzymatic activities involved in their own transposition processes.

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