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DNA polymerization by the reverse transcriptase of the human L1 retrotransposon on its own template in vitro

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Abstract **L1 elements (LINE-1s) account for 17% of the human genome and have achieved this abundance by transpositions via an RNA intermediate, or retrotransposition. Reverse transcription is a crucial event in the retrotransposition of the active human L1 element and is carried out by the L1-encoded ORF2 protein. Previously, we performed biochemical characterization of the human L1 ORF2 protein with reverse transcriptase (RT) activity (referred to as L1 RT), expressed in baculovirus-infected insect cells. In the present study, we describe the properties of DNA- and RNA-dependent DNA synthesis catalyzed by the L1 RT on the L1 templates in vitro. We found that L1 RT synthesized at least 620 of nucleotides per template binding event utilizing L1 RNA in vitro. Under processive conditions the L1 RT synthesized cDNA over 5 times longer than that Moloney murine leukemia virus RT on the heteropolymeric RNA template used in these studies. These data are the first to demonstrate that RT from the human L1 element is a highly processive polymerase among RT enzymes. This report also presents a strong evidence of lack of RNase H activity for the L1 ORF2 protein in vitro, distinguishing L1 RT from retroviral RTs. Finally, we found strong pausing for of the L1 RT during DNA polymerization within the 3′ untranslated region of L1 mRNA, that is result of contribution both rGs runs of the polypurine stretch and immediately adjacent stem–loop structure. A mechanism facilitating minus-strand DNA synthesis during reverse transcription of L1 element in vivo is discussed.**

Keywords: Non-LTR retrotransposons; LINE-1; Retroviruses; Reverse transcriptase; Processivity; Pausing

1. Introduction

The long interspersed elements (LINEs, L1s) are non-long terminal repeat (LTR) retrotransposons, which inhabit mammalian genomes. L1 elements account for 17% of the human genome and have achieved this abundance by transpositions via an RNA intermediate, or retrotransposition. Active L1 elements have the capacity to cause mutation, disease, genetic variation and polymorphism and their inactive copies appear to be involved in recombination and rearrangement [1]. Full-length human L1 elements are about 6 kb long and contain a 5′ untranslated region (UTR), two non-overlapping open reading frames (ORFs) and a 3′ UTR ending in a poly(A) tail [1]. The product of ORF1 is an RNA-binding protein, which associates with L1 RNA [2]. ORF2 encodes a protein of about 149 kDa, which has endonuclease [3] and reverse transcriptase [4] activities. The ORF2 polypeptide chain consists of N-terminal domain of AP-like endonuclease, reverse transcriptase domain and C-terminal domain of unknown function containing a putative zinc-binding motif [1]. L1-encoded proteins possess a *cis* preference, i.e. act preferentially on the L1 mRNA that encodes them [5]. The great majority insertions of L1s are 5′ truncated and transpositionally inactive [1,6], but the truncation mechanism is unknown up to now.

Reverse transcription is a crucial event in the retrotransposition of the human L1 element and is carried out by the L1 ORF2 protein. The cell culture-based retrotransposition assay studies have demonstrated that deletions and mutations in ORF2 inactivate retrotransposition in cultured human cells [7]. It has been shown that the L1 ORF2 protein uses the nicked DNA as a primer to initiate cDNA synthesis on the RNA template in target-primed reverse transcription (TPRT) reaction in vitro [8]. This mechanism of reverse transcription of non-LTR retrotransposons was originally demonstrated in experiments with reverse transcriptase encoded by the R2 element from *Bombyx mori* [9]. The experiments showed that R2 RT uses the free 3′ end at the target-site DNA nick to prime minus-strand DNA synthesis on the R2 RNA template in vitro. Lately, high proccessivity and template switching of DNA polymerization have been demonstrated for this reverse transcriptase [10,11]. Features of DNA synthesis catalyzed by the ORF2 protein of the human L1 element remain unclear so far.

Recently, we have expressed the human L1 ORF2 protein in baculovirus-infected insect cells and purified it using its reverse transcriptase activity (hereafter referred to as L1 RT). Additional biochemical studies have been performed on L1 RT [12].

In the present report, we describe the properties of DNA- and RNA-dependent DNA polymerization catalyzed by the L1 RT on the L1 templates in vitro. It has been observed that L1 RT synthesizes DNA on the minus ssDNA of element L1 effectively in vitro. We found that L1 RT polymerized at least 620 of nucleotides per template binding event utilizing its own RNA. Under processive conditions the L1 RT are able to synthesize cDNA much longer than that MMLV RT on the heteropolymeric RNA template used in these studies. These data demonstrated for the first time that RT of the human L1 element has higher processivity than that of most retroviral RTs. We also report that the L1 ORF2 protein displays a lack of RNase H activity in vitro, a facility that is required for all retroviral RTs. Moreover, we found strong pausing for of the L1 RT during DNA

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polymerization within the 3′ UTR of L1 mRNA, which is a result of contribution of both rG runs of the polypurine stretch and the immediately adjacent stem–loop structure. This mechanism, which facilitates minus-strand DNA synthesis during reverse transcription of L1 element in vivo is proposed here.

2. Materials and methods

2.1. Enzymes

The ORF2 protein with reverse transcriptase activity (L1 RT) of the active L1.2 retrotransposon used in this report is recombinant polymerase expressed in insect cells (Sf21) and purified as described in detail previously [12]. The specific RT activity of the purified L1 RT was about 400 U/μg protein determined as described earlier [12].

MMLV reverse transcriptase was obtained from Promega.

2.2. Templates and primers

The L1 sequence was derived from pSM42 (a gift of H.H. Kazazian) carried ORF2 and 3′ UTR of the human L1 element L1.2A [4,13]. The numbering of the L1.2 sequence is that of Genbank Accession No. M80343.

The DNA primer for the L1 DNA template was 26 nt oligonucleotide s-3′L1 (5′-TCCAACAATGATAGACTGGATGAAGA) corresponding to nucleotide positions 5629–5654 of L1 element. DNA primer for the L1 RNA templates was oligonucleotide a-L1-3′ UTR (5′-CGATTTCGAACCCTGACGTCT) complementary to pSM42-derived 20 nt sequence at 3′ end of the RNA templates.

RNAs spanning nucleotides 5674–6026 and 4838–6026 of L1 element were generated in vitro using the T7 transcription kit (Fermentas) from *Cla*I-linearized plasmids pT7-455 and pT7-1300 following extraction. Briefly, an 373 bp *Nco*I–*Cla*I fragment or 1209 bp *Bam*HI–*Cla*I fragment from pSM42 were cloned into the expression vector pT7-7 (USB) resulted in pT7-455 and pT7-1300, respectively, and RNA was transcribed from the T7 promoter. Synthesized 455 nt and 1294 nt RNAs carried a short vector-derived sequence at the 5′ end and pSM42-derived 20 nt at 3′ end. Both L1 RNA templates having the same 3′ end were hybridized with the DNA primer a-L1-3′ UTR.

The pT7-7 vector-derived 5′ terminus extremity of the RNA transcripts contained 21 nt self-complementary sequence directly at 5′ end, which was able to form a strong hairpin structure.

Single-strand L1 DNA from 3′ end of L1 element was generated by polymerase chain reaction (PCR) and enzyme digestion. At the first the 418 bp DNA fragment comprising of L1 3′ sequence (nt positions 5629–6026) was amplified by PCR from plasmid pSM42 using non-phosphorylated primer a-L1-3′ UTR (antisense) and primer s-3′L1 (sense), which was previously phosphorylated by T4 kinase. Following PCR amplification, the phosphorylated plus-strand of the PCR product was removed by digestion with lambda exonuclease (Fermentas). The minus-strand, which served as a template, was extracted with phenol/chloroform and precipitated with ethanol. The DNA primer s-3′L1 was annealed to the 3′ end of the generated 418 nt single-strand DNA template.

The primer–template complexes were prepared as follows: the primer and template were mixed at a 1:1 molar ratio, heated to 90 °C for 5 min, and then slowly cooled to room temperature for annealing.

2.3. Primer extension and processivity assays

Primer extension reactions were performed in 50 mM Tris–HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 1 U RNAsin and 20 nM template–primer. The L1 RT (12 ng) was added to either the DNA · DNA or the RNA · DNA template–primer, and the reaction mixtures were preincubated for 5 min at 37 °C. The DNA polymerization was initiated by addition of dNTPs, at a final concentration of 100 μM each dTTP, dCTP, dGTP and 50 μM dATP and 0.5 μl [α-^{32}P] dATP (~4000 Ci/mmol) in a total volume of 10 μl. Reaction mixtures were incubated at 37 °C for 20 min.

For processivity studies, either the L1 RT (12 ng) or the MMLV RT (40 ng) was preincubated with RNA · DNA template–primer for 5 min at 37 °C. The reactions were initiated by the addition of dNTPs with [α-^{32}P]dATP and an excess (2 μg) of poly(rC)/oligo$(dG)_{12-18}$ (Sigma) as a trap for RT and incubated at 37 °C for 20 min.

All polymerization reactions were stopped by addition of an equal volume of gel loading buffer. Reaction products were analyzed by electrophoresis in a 6% sequencing gels containing 7 M urea, and detected by autoradiography. The relative amounts of the primer extension products were determined by densitometric scanning of the gel autoradiographs. To determine the size of the RT products, sequencing reactions were carried out with the same primers and homologous DNA.

2.4. Assay for the RNase H activity of the L1 RT

The RNase H activity assay was performed by following the cleavage the RNA portion of an RNA/DNA hybrid. The internally [α-^{32}P]-labeled 455 nt RNA, synthesized in vitro using the T7 transcription kit (Fermentas) from the pT7-455 plasmid linearized by *Cla*I, was annealed to the complementary 39 nt long synthetic DNA oligonucleotide at a molar ratio of 1:1. The reaction mixture contained 50 mM Tris–HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 1 U RNasin, a 0.1 pmol RNA/DNA hybrid and the L1 RT (concentrations described in Fig. 4) in a total volume of 10 μl. The samples were incubated for 1 h at 37 °C. The reaction was stopped by addition of an equal volume of formamide gel loading buffer. The RNA cleavage products were analyzed by electrophoresis in 8% denaturing polyacrylamide–7 M urea gel, and detected by autoradiography. MMLV RT with RNase H activity (Promega) was used as a positive control.

3. Results

3.1. Primer extension by the L1 RT on the L1 DNA and RNA templates

Purified recombinant L1 RT was examined for its capacity to synthesize DNA on DNA and RNA templates derived from the 3′ end portion of the human L1 element in primer extension assay in vitro. Fig. 1 shows the size of DNA products synthesized by DNA- and RNA-dependent DNA polymerase activity of L1 RT on the templates. The DNA template for this reaction was a 418 nt minus-strand DNA molecule of L1 3′ end with 26 nt DNA primer annealed. We found that L1 RT generated a single extended DNA product, whose length corresponded to that of L1 DNA template (Fig. 1B, lane 1). For the RNA template, a 455 nt RNA molecule, containing the L1 3′ end of L1 element with 21 nt DNA primer annealed was used. The primer for the initiation of the reverse transcription reaction on the L1 RNA transcripts was designed to initiate DNA elongation of the template starting from an rA (Fig. 3). Fig. 1B, lane 2, shows that L1 RT generated a range of extension cDNA products, the largest of which (at 455 nt), matched that of the RNA template used. These results demonstrate that, the L1 RT is able to extend the primers efficiently and to synthesize high molecular weight products on DNA as well as RNA templates of the L1 element.

3.2. Highly processive DNA synthesis by L1 reverse transcriptase

The processivity of a DNA polymerase can be defined as the number of nucleotides incorporated in polymeric form before the enzyme dissociates from the template–primer complex. Primer extension on the L1 RNA transcripts was performed using an excess competitive substrate to determine the processivity of RNA-dependent DNA synthesis catalyzed by L1 RT. Processivity assays containing commercial MMLV RT were included for comparison studies. The cDNA polymerization was carried out both in the absence and presence of an excess poly(rC)–oligo(dG) as a competitive substrate. This trap for RT molecules was added after incubating RTs with the RNA/primer complex and prior to the addition of the labeled dNTPs mix. Hence, the