Detection and cloning of LINE-1 elements in CHO cells

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Abstract Long interspersed elements (LINEs, L1s) are non-long terminal repeat (LTR) retrotransposons found in mammalian genomes and account up to 20% of genomic DNA. It has been shown that active L1 elements can cause mutation resulting in disease, genetic variation and polymorphisms and their inactive copies seem to be involved in recombination and rearrangement. L1-encoded products have been detected in a number of tissues including mammalian germ cell tumours, breast carcinomas and a large variety of transformed mouse and human cell lines. Chinese Hamster Ovary (CHO) cells are widely used in the manufacture of recombinant proteins for biopharmaceuticals. Here, we investigated the transcriptional activity of hamster L1 elements in CHO-K1 cells. These cells were analysed for the presence of L1 RNA transcripts. The sequence, which is homologous to mammalian L1 elements, was cloned from hamster genomic DNA and used to design primers for RT-PCR. L1 transcripts were detected in CHO-K1 RNA.

Keywords L1 elements · Retrotransposon · Transcripts · CHO-K1

Introduction

Long interspersed elements (LINEs, L1s) are non-long terminal repeat (LTR) retrotransposons, which account for up to 20% of the mammalian genome. L1 elements have achieved this abundance by transposition via an RNA intermediate, or retrotransposition. The consensus full-length L1 element is between 6–7 kb long and contains both 5′ and 3′ UTRs, and two open reading frames (ORFs) (Ostertag and Kazazian 2001). Active L1 elements have the capacity to cause mutation resulting in disease, genetic variation and polymorphisms and their inactive copies appear to be involved in recombination and rearrangement. L1-encoded proteins have been detected in the cytoplasm of human testicular germ cell tumours, in breast carcinomas and medulloblastomas and also in mouse embryonal carcinomas cell lines, male and female mouse germ cells, Leydig cells of embryonic mouse testis, theca cells of adult mouse ovary, and a large variety of transformed mouse and human cell lines (Ostertag and Kazazian 2001).

Chinese Hamster Ovary (CHO) cells are the most widely used mammalian cell line in the Biopharmaceutical industry. This industry is highly regulated to ensure product safety and ultimately patient well-being. Any drug produced from a non-human source must demonstrate freedom from potential zoonotic agents, including

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viruses. It has been shown that endogenous retroviral-like sequences, related to type C and Intracisternal A Particle (IAP) genes, in the Chinese hamster genome may be transcriptionally active. Products of these retroviral elements have also been detected in the cell cytoplasm and culture medium (Anderson et al. 1991; Emanoil-Ravier et al. 1991; Dinowitz et al. 1992; de Wita et al. 2000). This has consequences for recombinant protein production and, in particular, downstream purification procedures.

Another concern during recombinant protein expression is genomic stability and integrity of the producer cell line. Though usually attributed to transgene amplification due to selection conditions causing genomic re-arrangements, the role of repeat regions, such as L1s, has not yet been considered.

The hamster genome has not been sequenced, or at least the information is not in the public domain. Moreover, GeneBank contains no hamster non-LTR retrotransposon sequences. In order to obtain the hamster L1 sequence we used an approach based on analyses reported by Eickbush and colleagues (Malik et al. 1999; Xiong and Eickbush 1990). They performed a comprehensive phylogenetic analysis of all non-LTR retrotransposons based on alignment of their conserved domains, such as endonuclease, reverse transcriptase (RT) and RNaseH. The search showed that the RT domain is the most conservative part of the L1 element. Mouse and rat RT domain sequences are the most similar. As the hamster belongs to rodent family, alignment of mouse and rat L1 ORF2 sequences were used to design primers to amplify L1 sequences from hamster genomic DNA. We also investigated the presence of L1 RNA transcripts in the CHO-K1 cell line.

Materials and methods

Cell culture and extraction

CHO-K1 cells were obtained from the NICB culture collection and maintained in DMEM/Ham's F12 supplemented with 10% FCS. Genomic DNA was isolated from CHO-K1 cells using a

DNA isolation kit (Nucleon). Mouse genomic DNA (Promega) was used as a control. Total RNA was isolated from CHO-K1 cell line using RNeasyTM extraction kit (Qiagen) according to the manufacturers instructions. Human total RNA was used as a control (GeneRacerTM kit, Invitrogen). All oligonucleotides used in this study were synthesized by MWG Biotech, Germany.

Cloning L1 elements

Primers were designed based on conserved regions of L1 ORF2 from Tf subfamily L1Md-Tf30s sequence (Genbank Accession No. AF081114). The reverse primer contained a unique XhoI site for cloning. The sequences of the oligonucleotides and their positions are shown in Table 1. PCR amplification of genomic DNA was performed with 80 ng DNA, 0.2 μ M each forward and reverse primers in 20 mM Tris, pH 8.4, 2.5 mM MgCl₂, 0.25 mM deoxynucleotides, and 2.5 U *Taq* DNA polymerase. Predenaturation at 95 °C for 5 min, then 10 cycles of 92 °C for 2 min, 50 °C for 3.5 min and 72 °C for 3.5 min, followed by 20 cycles of 92 °C for 2 min, 58 °C for 3.5 min, and 72 °C for 3.5 min. Final elongation was at 72 °C for 4 min. PCR products were separated on agarose and purified with the QIAquick gel extraction kit (Qiagen), digested with XhoI and cloned into pBluescript vector (Stratagene). Plasmid preparation, bacterial transformation (JM109) and all other molecular biological techniques were performed according to standard procedures described by Sambrook et al. (1989). DNA sequencing was performed on both strands (MWG).

RT-PCR and 3' RACE

RT-PCR primers were design to anneal to the conserved RT region based on the sequence cloned from hamster genomic DNA. The following primers were used: RT-1_for 5'-CAAGGCTGTCCACTCTCTCCATACCTC-3'; RT-2_for 5'-CATCTTCAGCAAAGTGGCAGGATACAAG-3'; RT_rev 5'-CTTGTATCC TGCCACTTTGCTGAAGATG-3'. RT-PCR was performed using the GeneRACERTM kit (Invitrogen) that included control RNA template

Table 1 List of oligonucleotides producing DNA fragments in PCR with the hamster genomic DNA

Oligo	Sequence	mer	Position (nt)	PCR product (kb)
Olpi-2_rev	5'-AAAAAACTCGAGCCATTGTGTAGATGTACCAC-3'	32	5,841–5,861	
Olpi-3_for	5'-GGGACTAGACAAGGCTGCCC-3'	20	4,141–4,161	1.7
Olpi-7_for	5'-ATGCCACCTTTAACAACATAACA-3'	29	2,155–2,184	3.7
Olpi-8_for	5'-CCTCACACAATAATAGTGGGAGACTTC-3'	27	2,587–2,614	3.3
Olpi-9_for	5'-ACCTTCTTCTCAGCACCTCATGG-3'	23	2,749–2,772	3.1
Olpi-10_for	5'-GCAGCTTGACAACACATCTAAAAGCTC-3'	27	3,119–3,146	2.7
Olpi-11_for	5'-CCCAGGACCAGACGGGTTTAGTGC-3'	24	3,585–3,609	2.3

Each sense primer (_for) was used in conjunction with the same antisense primer (Olpi-2_rev). Position refers to mouse sequence

and primers (beta-actin). Touchdown PCR was used to amplify cDNA. The conditions were as follows: 1 cycle of 94 °C for 2 min; 5 cycles of 94 °C for 30 s and 72 °C for 1 min; 5 cycles of 94 °C for 30 s and 70 °C for 1 min; 25 cycles of 94 °C for 30 s, 65 °C for 30 s and 68 °C for 1 min; and 1 cycle of 68 °C for 10 min.

Results

Full alignment of L1 elements from different species followed by phylogenetic analysis by Eickbush et al. (Malik et al. 1999; Xiong and Eickbush 1990), demonstrated that the RT domain sequence could be used to establish relation between species. Mouse and rat L1 elements were found to possess the highest similarity and

were placed in the same phylogenetic group (Malik et al. 1999). Amino acid alignment of the RT domain identified seven conserved regions common to all retroelements (Xiong and Eickbush 1990). Our experimental design is shown in Fig. 1. Bearing in mind that the hamster is closer to rat and mouse than human in evolutionary terms, the alignment of the mouse L1 ORF2 (AF081114) with the rat L1 ORF2 (U83119) was used to search for regions of high homology of approximately 30 nt in length. In total, 16 primers were designed: 13 forward and three reverse.

PCR was performed and optimised to amplify products from genomic DNA using these primers. Each of the three reverse primers was examined in combination with all forward primers. Mouse genomic DNA was used in control reactions. PCR products were observed in all reactions with

Fig. 1 Strategy for PCR amplification of L1 sequences from genomic DNA. **(A)** Structure of mammalian L1 element from 5' to 3' (Scale is kb). Open reading frames (ORFs) and untranslated regions (UTRs) are indicated by grey and black rectangles, respectively. **(B)** Schematic amino acid sequence alignment of rat and mouse L1 ORF2 sequences. Sources of elements and database accession numbers: L1*Rn*, rat (U83119); L1*Md*, mouse (AF081114). Seven peptide regions (Ostertag and Kazazian 2001;

Anderson et al. 1991; Emanoil-Ravier et al. 1991; Dinowitz et al. 1992; de Wita et al. 2000; Malik et al. 1999; Xiong and Eickbush 1990) which are common for retroelements are highlighted by empty rectangles. **(C)** Primer design for cloning L1 sequence from hamster genomic DNA. DNA alignment of rodent L1 ORF2 sequences. Conserved regions are indicated by striped bars. Location of oligonucleotides producing DNA fragment in PCR and listed in Table 1 are shown by arrows

Fig. 2 PCR amplification of L1 sequences from genomic DNA, lane 1: DNA markers; lanes 2–7: hamster genomic DNA; lanes 8–13: mouse genomic DNA; lanes 2 and 8: olpi-3_f; lanes 3 and 9: olpi-7_f; lanes 4 and 10: olpi-8_f; lanes 5 and 11: olpi-9_f; lanes 6 and 12: olpi-10_f; lanes 7 and 13: olpi-11_f. Primer Olpi-2_rev common to all reactions

mouse genomic DNA, but only in six reactions with hamster genomic DNA. The sequences of these forward and reverse oligonucleotides are shown in Table 1 and corresponding PCR results in Fig. 2. Only three forward primers yielded a product of the size anticipated (based on the mouse sequence) when hamster DNA was used as template. In these reactions olpi-3_for and olpi-2_rev generated a 1.7 kb fragment, olpi-9_for and olpi-2_rev resulted in a 3.1 kb fragment, and olpi-11_for and olpi-2_rev resulted in a 2.3 kb fragment. The other three forward primers resulted in PCR products shorter than expected. The sequence of 1.7 kb, 2.3 kb and 3.1 kb PCR fragments were cloned and determined.

Next these sequences were compared amongst each other and with known rodent L1 sequences.

Alignment of the cloned hamster L1 sequences with mouse and rat sequences demonstrated 80% and 72% similarity, respectively (Fig. 3). Based on the clone alignments, we generated putative consensus sequence of a 1.7 kb fragment of the hamster L1 element. This region corresponds to nt positions 4100–5861 of L1 from *Mus musculus* (Genbank accession no AF081114) and is part of the ORF2 containing the RT domain. This sequence was used to design new sets of oligonucleotides for analysis of total RNA from CHO-K1 cells.

To determine whether L1 transcripts could be detected in CHO-K1 cells RT-PCR was performed on total RNA with different sets of primers. All primers were designed to anneal to the conserved RT region. The first set, RT-1_for and RT_-rev, should amplify a 350 bp fragment. Two other sets, RT-1_for and RT-2_for paired with an oligo(dT) primer should produce a 2.0 kb and 1.65 kb PCR fragment, respectively. As can be seen, primers RT-1_for and RT_rev produced a PCR fragment of the expected size (Fig. 4, lanes 5 and 6). RT-1_for and oligo(dT) gave two bands, neither of the expected 2.0 kb (Fig. 4, lane 4). RT-2_for and Oligo(dT) primers produced one fragment which was also shorter than expected (Fig. 4, lane 8). A control PCR on RNA with RT-1_for and RT_rev (no reverse transcription) gave no band, demonstrating absence of DNA contamination in the RNA samples.

Fig. 3 Alignment of cloned hamster L1 sequence with known rodent L elements, (A) Structure of mammalian L1 element from 5' to 3'. Open reading frames (ORFs) and untranslated regions (UTRs) are indicated by grey and black rectangles, respectively. (B) Alignment of rodent L1 sequences. Sources of elements and database accession numbers: L1Rn, rat (U83119); L1Md, mouse (AF081114),

L1Cg, Chinese hamster. Determined DNA sequence marked by solid line, undetermined by dotted line. Similarity level is shown by intensity of striped bar. The conserved region corresponding to RT domain is indicated by empty rectangle. Presence of FADD amino acid sequence necessary for RT activity is indicated

Fig. 4 RT-PCR of RNA extracted from CHO-K1 cells, lane 1: DNA markers; lane 2: Control primer A and Oligo(dT) primer amplifying full-length beta-actin gene (1.8 kb) from human RNA; lane 3: Control primer A and oligo(dT) + hamster RNA; lane 4: RT-1_for and oligo(dT) + hamster RNA; lane 5: RT-1_for and RT_rev + hamster RNA; lane 6: RT-1_for and RT_rev + hamster RNA; lane 7: RT-2_for and oligo(dT) + hamster RNA without RT step; lane 8: RT-2_for and oligo(dT) + hamster RNA

Discussion

This work aimed to investigate whether active L1 elements could be detected in CHO-K1 cells and to compare the sequence to other species. The presence of either L1 transcripts or proteins has not been reported previously in CHO—the ‘workhorse’ cell line in the production of biopharmaceuticals.

The DNA sequences of these elements are highly conserved and homologous within species. The proteins encoded by the second frame of the L1 element from different species share the same conserved amino acid sequences (Malik et al. 1999; Xiong and Eickbush 1990). For this reason, L1 elements have been used as tools in different phylogenetic analyses (Malik et al. 1999; Xiong and Eickbush 1990; Boissinot et al. 2000).

A search for any hamster L1-like sequences in GenBank revealed none. Instead we cloned the L1 sequence from hamster genomic DNA. The design of primers for PCR was based on the presence of highly conserved domains and regions in L1 elements by aligning mouse and rat ORF2. Four primers out of 16 generated three PCR products with the expected size, based on the mouse sequence, and high yield. By comparison of the nucleotide sequences of the cloned fragments, a 1.7 kb consensus sequence was generated which had high similarity with the 3′ portion of rodent L1 elements, 80% for mouse and 72% for rat. This

1.7 kb fragment contained part of the hamster L1 ORF2 including the conserved RT domain and the FADD amino acid group responsible for RT activity. The RT activity of the ORF2 protein is responsible for copying the L1 RNA template into DNA (Piskareva and Schmatchenko 2006) and propagation of these elements in genome (Moran et al. 1996). Thus we have demonstrated the presence of the RT DNA sequence in the hamster genome. We would expect that the full-length hamster L1 element would have the same structure as other mammalian L1s.

RT-PCR analysis of RNA extracted from CHO-K1 cells revealed the presence of actively transcribed L1 mRNA. This finding is not unexpected given that transcripts for these elements have been reported previously, particularly in malignant tissue or transformed cell lines (Oster-tag and Kazazian 2001). Primer design was based on the cloned 1.7 kb genomic sequence to amplify fragments of the RT region.

In an attempt to extend the cloned sequence further, we performed amplification of 3′ ends of L1 RNA with L1-specific primers. This method generated fragments of hamster L1 RNA but of shorter size than expected. The reasons for this are not yet clear. The oligo(dT) primer used in the reaction consisted of a poly-T ending in a specific 36 nt DNA sequence. A BLAST search of this sequence in Genbank revealed no matches with retroviral elements. It is possible that priming may have occurred at short internal poly-A sites. Bearing in mind that the cloned genomic sequence corresponded to the mouse L1 element in terms of length (Fig. 3), a possible explanation is that one or more truncated L1 elements are active in CHO cells. This would be consistent with the nature of these elements being frequently rearranged in the genome, including partial deletions, duplications and other mutations (Malik et al. 1999). It is still possible that tiny amounts of full-length hamster L1 RNA are expressed in CHO-K1 cells but we were unable to detect any. Examination of different cell lines of hamster origin may yet yield full-length L1 RNA. Identification of the full coding sequence of these elements will facilitate the search for the presence of translated proteins of L1 origin in these cells.

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