A potential role for the nucleolus in L1 retrotransposition

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Determining the subcellular localization of the L1 ORF2 protein (ORF2p) has been impossible to date because of technical limitations in detecting either endogenous or overexpressed forms of the protein. Here we report visualization of the full-length ORF2p in cultured human cells following expression in a modified vaccinia virus/T7 RNA polymerase (MVA/T7RP) system. The MVA/T7RP system was used to ascertain subcellular localization of L1 ORF1p and ORF2p both as fusions with green fluorescent protein and by immunocytochemistry. Full-length ORF2p was predominantly cytoplasmic, while carboxy-terminal-deleted ORF2p localized additionally to the nucleolus. We mapped a functional nucleolar localization signal in ORF2p. ORF1p appeared in the cytoplasm with a speckled pattern and colocalized with ORF2p in nucleoli in a subset of cells. These findings help explain the presence of chimeras between L1s and small RNA gene sequences recently discovered in the human genome.

INTRODUCTION

Retrotransposons are divided into two major types: retroviral-like or long terminal repeat (LTR) retrotransposons, and LINEs (long interspersed nucleotide elements) or non-LTR retrotransposons. Both types retrotranspose in a replicative manner involving reverse transcription of an RNA intermediate and insertion of its cDNA copy at a new site in the genome. However, the mechanisms of transposition differ fundamentally between the two types.

Roughly 520 000 LINE-1 (L1) copies occupy 17% of the human genome (1). These L1s have mediated significant genomic modifications, including transduction of flanking DNA to new locations, deletion of sequence at sites of insertion, recombination and insertional mutation (2,3, reviewed in 4,5). De novo retrotransposition events, of which 16 in humans and seven in mice have been characterized, can cause disease (4). Although most L1s are inactive due to mutation, 5' truncation, or sequence rearrangement, it is estimated that 80–100 L1s remain potentially active in an individual genome (6).

As autonomous retrotransposons, L1 elements encode proteins necessary for their own mobilization. A 6.0 kb full-length human L1 (Fig. 1A) has a 906 nt 5' untranslated region (UTR), two open reading frames (ORF1 and ORF2) separated by a 63 bp intergenic spacer, and a 206 nt 3' UTR which ends in a poly(A) signal and tail. The 5' UTR functions as an internal promoter directing transcription across the length of the element (7).

Insights into mechanisms of L1 retrotransposition derive largely from promoter studies, a cell culture assay for retrotransposition and examination of endogenous genomic insertions (reviewed in 5). A model for L1 retrotransposition, called 'target primed reverse transcription', originates from studies of the site-specific R2 retrotransposon of arthropods (8). According to this model, element-encoded endonuclease nicks the bottom strand of target DNA and exposes a 3' hydroxyl which primes reverse transcription of the element's RNA. Recently an in vitro TPRT assay has been reported for human L1 using recombinant ORF2 protein (9).

Compared with the exquisitely detailed studies of some LTR retrotransposons, Group II introns and DNA transposons, our understanding of L1 protein function is limited. Most attention has focused on the 40 kD ORF1 protein (ORF1p). While mutational analysis has shown ORF1p is required for retrotransposition (10), its exact role is unclear, although it forms multimeric complexes and can function as a nucleic acid chaperone (11,12). ORF1p has been detected in the cytoplasm of human and mouse carcinoma and germ cells and has been isolated as ribonucleoprotein (RNP) particles in association with L1 RNA (13–15).
L1 ORF2 encodes a 149 kD protein (ORF2p) having endonuclease (EN) and reverse transcriptase (RT) activities, and a C-terminal cysteine-rich motif (CX3CX7HX4C). Unlike ORF1p, translation of ORF2p is suppressed, and there are no published reports of endogenous or overexpressed ORF2p detected in tissues or cultured cells (5,16). Difficulties of detection have hampered functional analyses. To overcome this limitation, we exploited the modified vaccinia virus Ankara/T7 RNA polymerase (MVA/T7RP) hybrid expression system (17,18) to transiently boost expression of ORF2p to detectable levels in both primary and immortalized cultured cells. Both ORF1 and ORF2 proteins are predominantly cytoplasmic, with co-localization in nucleoli in a subset of positive cells. A functional nucleolar localization signal maps to the EN domain of ORF2p. By overcoming technical barriers for ORF2 protein detection, this study reveals aspects of L1 biochemistry which further our understanding of L1 propagation.

RESULTS

MVA/T7RP-mediated expression of L1 proteins in primary and transformed cells

The MVA/T7RP expression system permits high protein expression levels in cells hundreds of fold in excess of conventional eukaryotic expression systems (18–21). Since we were unable to detect ORF2p expression by these more conventional approaches, including inducible systems in mammalian and yeast cells (unpublished data), we turned to the MVA/T7RP system. Initially, FLAG epitope-tagged ORF2 sequence from L1.2A (Fig. 1B), an L1 competent for retrotransposition in cell culture (10,22) significantly increased expression of ORF2p, as shown in human osteosarcoma 143B TK-cells (Fig. 2C, lane 3) and confirmed in HUVECs (data not shown) (143B TK-cells were selected for study because they support the highest known rate of L1 retrotransposition). Interestingly, a second mutation in the reverse transcriptase domain further increased protein levels (Fig. 2C, lane 2). A cryptic nuclease activity reported in the RT domain (9) may account for some cytotoxicity.

Both full-length and truncated forms of ORF2p were detected in 143B cells by α-ORF2-N or α-FLAG antibodies (Fig. 2C, lanes 2, 3 and 5). The largest product had the same apparent molecular weight as tagged-ORF2p generated by baculovirus in insect cells (Fig. 2C, lane 6). An antibody against sequence at the extreme carboxy-terminus (α-ORF2-C) detected only the full-length product (Fig. 2C, lane 8). This is consistent with truncated ORF2p forms lacking a carboxy-terminus due to either premature termination of T7RP or cleavage by a cellular protease. Interestingly, proteolysis of recombinant ORF2p augments in vitro endonuclease activity normally repressed in the full-length protein (9).

Full-length ORF2p is cytoplasmic but truncated ORF2p is both cytoplasmic and nucleolar

Immunocytochemistry was performed on fixed 143B cells to determine subcellular localization of ORF2p produced in the MVA/T7RP system. Brightly fluorescing cells expressing ORF2p were detected by α-ORF2-N (Fig. 3), α-ORF2-C and α-FLAG antibodies (data not shown). Faint background fluorescence of non-transfected cells was the same whether exposed to α-ORF2 antibodies or preimmune sera, indicating that endogenous L1 protein was not detected.

ORF2 protein was cytoplasmic when assayed with α-ORF2-N or α-FLAG antibodies. Nuclear staining was absent or faint, but nucleolar concentration of ORF2p occurred in 65% of non-mitotic cells (Fig. 3A and Table 1). Nucleolar localization was confirmed by communostaining with patient sera expressing anti-nucleolar antibodies (α-ANA-N, Fig. 3B). No difference
in localization existed between wild-type ORF2 and FLAG-tagged H230A/D702Y mutant ORF2, although many fewer cells expressed wild-type protein (Table 1). Interestingly, α-ORF2-C detected ORF2p in nucleoli of only 5% of cells. Furthermore, nucleolar localization of ORF2p fused with C-terminal GFP was greatly reduced (17%) over that seen for N-terminal GFP fusions (88%). Together, these data indicate that ORF2p truncated at the C-terminus is preferentially retained in nucleoli.

**ORF2 EN contains a functional nucleolar localization signal**

To identify potential nucleolar localization signals (NoLSs), we created constructs containing GFP fused with 100-amino acid fragments spanning the length of ORF2. Protein localization was examined by fluorescence microscopy of living HeLa cells without MVA infection. Two fragments, residues 1–100 and 1001–1100, directed GFP to nucleoli. Microdeletion mapping of the 1–100 fragment identified a highly basic region (34%) between residues 50 and 93 which still directed GFP to nucleoli (Fig. 3D–G). The 1000 to 1100 fragment also contains a highly basic stretch of 33 residues. NoLSs and nuclear localization signals (NLSs) are both enriched in basic amino acids and the two signals often overlap (23). However, no NLS was predicted in ORF1 or ORF2 by the PredictNLS server (http://cubic.bioc.columbia.edu/predictNLS/) (24).

Eight basic residues between residues 53 and 71 of FLAG-tagged mutant ORF2 were replaced with alanine. Nucleolar localization decreased from 65 to 14% of non-mitotic cells with no change in cytoplasmic staining (Table 1), confirming the presence of a functional NoLS in the EN domain. However, when ORF2p lacking residues 1001 to 1275 and the putative C-terminal NoLS was expressed, there was minimal change in nucleolar localization frequency, indicating that this signal is weak or masked.

A comparison of human ORF2 with ORF2 sequences of LINE elements from other species (including mouse, rat, rabbit, slow loris and medaka) reveals conservation in the percentage and approximate position of basic residues in the region corresponding to the human N-terminal NoLS (data not shown). This suggests that this motif may be important for retrotransposition in other species.

**ORF1p is also detected in the nucleolus by immunofluorescence**

We also expressed ORF1 with N- or C-terminal GFP tags in living HeLa and 143B cells and found the protein to be cytoplasmic with 40% of cells also having speckled patterning (Fig. 4A). No nucleolar localization was observed. Martin and colleagues also reported punctate cytoplasmic staining of endogenous mouse ORF1p in F9 cells (25). ORF1p was not associated with the Golgi apparatus, lysosomes or endoplasmic reticulum, and they proposed that the speckles were RNP aggregates.

Red fluorescent protein-tagged ORF1 was also strictly cytoplasmic when assayed in fixed cells in the MVA/T7RP system (Table 1). However, C-terminal T7-tagged ORF1p was not only cytoplasmic but also nucleolar in 46% of fluorescent cells (Table 1 and Fig. 4B–G). N-terminal FLAG-tagged ORF1p was also detected in nucleoli. One explanation for this discrepancy is that ORF1p does not contain a NLS, and when fused with a fluorescent protein is too large (68 kD) to diffuse into the nucleus (26). However, epitope-tagged monomeric ORF1 is small enough to diffuse into the nucleus and concentrate in nucleoli by binding to resident macromolecules. Although it has been demonstrated that human ORF1p forms multimers (14), and that recombinant mouse ORF1p forms

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**Table 1. Frequency of nucleolar localization of L1 proteins in non-mitotic human 143B TK-cells**

<table>
<thead>
<tr>
<th>Construct name</th>
<th>Antibody (+α-ANA-N)</th>
<th>No. of cells counted</th>
<th>% of cells nucleolar</th>
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<tr>
<td>ORF2p only</td>
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<td></td>
<td></td>
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<tr>
<td>pMINI-FLAG-ORF2 WT</td>
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<td>5</td>
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<td>225</td>
<td>14</td>
</tr>
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<td>53</td>
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<td>α-ORF2-N</td>
<td>473</td>
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<td>98</td>
<td>88</td>
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<tr>
<td>Both proteins</td>
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**FLAG:** N-terminal FLAG-tag; **WT:** wild-type; **H230A:** EN domain mutation; **D702Y:** RT domain mutation; **modNoLS:** nucleolar localization signal mutation; **-GFP:** C-terminal green fluorescent protein tag; **GFP-:** N-terminal green fluorescent protein tag; **-RFP:** C-terminal red fluorescent protein tag; **-T7:** C-terminal T7-tag.
stable trimer complexes which could not passively enter the nucleus, a pool of monomeric ORF1p may exist in the cytoplasm, at least when overexpressed.

We also considered that L1 proteins might possess nuclear export signals (NESs) causing active transport to the cytoplasm. An NES usually contains a short stretch of hydrophobic residues, typically leucine, but less frequently isoleucine, valine, methionine or phenylalanine. Glutamic acid and aspartic acids are also favored (27). Putative NESs from ORF1 and ORF2 (see Materials and Methods) were fused with GFP and expressed proteins were examined by fluorescence microscopy in living 143B cells. A functional NES should cause GFP, which is normally both cytoplasmic and nuclear, to become predominantly cytoplasmic (28). No changes in GFP localization were detected (data not shown). Furthermore, no increase in nuclear accumulation of either ORF1p or ORF2p was detected following incubation with LeptomycinB, an inhibitor of nuclear export factor CRM1 (29). We conclude that L1 proteins do not possess functional leucine-rich NESs.

**ORF1p and ORF2p colocalize**

We expressed in the MVA/T7RP system a bicistronic construct containing ORF1 and ORF2, separated by their intergenic spacer and followed by 150 bp of L1 3' UTR. ORF1p was easily seen and its subcellular localization was the same as when the protein was expressed alone. No ORF2p was detected, however. When this bicistronic construct was assayed in the TnT T7 Coupled Reticulocyte Lysate System (Promega) using [35S]methionine-radiolabelling and electrophoretic separation, ORF2p expression was detected, but at a level 0.1% or less that of ORF1p (data not shown). In contrast, placing an IRES at the beginning of each ORF contained in a single construct, allowed detection by immunofluorescence of both ORF1p and ORF2p in a single cell (Table 1).

Distribution of both proteins was predominantly cytoplasmic, with ORF2p often, but not always, coexisting with ORF1p aggregates (Fig. 4H–M). The frequency of nuclear localization of ORF1 was similar whether expressed alone or with ORF2p (46% versus 42%), and nucleoli of only 52% of cells positive for ORF2p also contained ORF1p. These two facts indicate that ORF1 and ORF2 proteins either do not directly bind each other or bind transiently.

**DISCUSSION**

The MVA/T7RP system is an efficient strategy for transient expression of L1 ORF2 protein in both primary and tumorigenic cells. Western blotting unequivocally detects the protein without need for concentration by immunoprecipitation or column chromatography. It has also been possible to assay ORF2p subcellular localization both alone and when coexpressed with the ORF1 protein product. Furthermore, the ability to generate easily detectable quantities of ORF2 protein in human cells now makes feasible experiments dealing with protein modification, interaction with other cellular proteins, translation control and other currently unknown aspects of L1 biochemistry.

As with all overexpression approaches, ectopically-produced proteins may differ functionally from their endogenous counterparts. It is possible the L1 proteins produced by the MVA/T7RP hybrid system differ in cellular localization and behaviour from those produced from their own promoter and genomic loci. However, vaccinia virus/T7RP hybrid expression systems have been used to overexpress a variety of proteins, including those with different cellular localizations, activities and protein–protein requirements, without apparent artifactual effects on activity (21,30–32 and unpublished data). Furthermore, employing the MVA/T7RP system, we have examined by immunofluorescence several non-L1 proteins, including GFP, CDK4 and RanGap1, and found these to have expected subcellular distribution without nucleolar concentration (data not shown).

Our data suggest that a nucleolar localization signal in the EN domain of ORF2 protein is suppressed by interaction with the C-terminus. Cytoplasmic localization is probably maintained by the large size of the protein, which cannot enter the nucleus by passive diffusion. Loss of a C-terminal fragment allows truncated ORF2p to move to the nucleolus.

Translation of endogenous ORF1 and ORF2 proteins from a bicistronic L1 is likely coupled. Also, evidence suggests that both proteins possess strong cis preference, tending to mobilize their own encoding RNA. Protein activity in trans occurs at low but detectable frequency and likely has been responsible for propagating the many Alu retroelements and processed
pseudogenes present in the human genome (33–35). In this study we examined subcellular localization of ORF1p and ORF2p both in isolation and together when expressed from an RNA transcript having an IRES at the beginning of each ORF. To date we have been unable to couple protein production by expressing both ORFs from a wild-type L1 transcript at a level where ORF2p can be detected. Therefore, we cannot say that subcellular distribution detected for these proteins in isolation is the same as when bound within an L1 RNP. Confirmation of nucleolar trafficking during active retrotransposition must await improved methods for isolating L1 RNPs and for tracking their movement in cells.

Why would trafficking to the nucleolus be a part of L1 retrotransposition? In addition to ribosome biogenesis, the nucleolus is a site of RNP assembly and maturation for many viruses and telomerase (36–38). Perhaps this is also the case for L1 RNPs. Phylogenetic analyses have revealed evolutionary relationships between non-LTR retrotransposons and RNA viruses (39). Furthermore, the reverse transcriptase catalytic subunit of telomerase, which contains a nucleolar signal, is phylogenetically related to non-LTR retrotransposons (40,41). Interestingly, one class of non-LTR elements, the site-specific R1 and R2 retrotransposons of arthropods, have evolved to insert into 28S rRNA gene sequences at nucleoli (42). Moreover, the integrase of Ty3, a yeast LTR retrotransposon, targets the nucleolus (43). Thus, L1 proteins, by entering nucleoli, preserve a leitmotif which permeates the world of RNA-mediated mobile elements.

Transit of retrotransposition-competent L1 RNPs through nucleoli may explain the unexpected discovery of chimeras between L1s and small RNA gene sequences. A family of chimeric repeats consisting of full-length copies of U6 cDNA fused to the 5′ ends of truncated L1 sequences was identified in the human genome (44). Subsequent analyses revealed other chimeric retroelements, including L1 fused with U3, U5 and 5S sequences, as well as 5S-Alu, 7SL-Alu, Alu-mRNA and U6-mRNA hybrids (45). All bore hallmarks of L1-mediated retrotransposition including poly(A) tails and TSDs. All of these small RNA species enter the nucleolus (46–50) and perhaps it is here they are captured in L1 RNPs and carried to the site of reverse transcription and genome insertion.

MATERIALS AND METHODS

Cloning

A 3′ SaI site and a 5′ NcoI site followed by a FLAG-tag were introduced into ORF2 of the active element L1.2A (GenBank no. M80343) (51) using PCR techniques. This fragment was cloned into both the MVA/T7RP vector, pMINI, to create pMINI-FLAG-ORF2 WT, and the baculovirus expression vector, pFASTBac1 (Invitrogen), to create pFAST-FLAG-ORF2 WT. pFAST-FLAG-ORF1 was generated in a similar manner and all clones were sequenced in their entirety.
To generate mutants, pMINI-FLAG ORF2 H230A and pMINI-FLAG-ORF2 H230A/D702Y, fragments from constructs previously described (10,22) were swapped into pMINI-FLAG-ORF2 WT. The EN-domain NoLS of pMINI-FLAG-ORF2 H230A/D702YmodNoLS contained the following residue changes, 53-(R/A)L(K/A)I(K/A)GW(RK/AG)IYQANG(K/A)Q(KK/AA)-71. pMINI-FLAG-ORF2 H230A/D702Y1001–1275 is a construct deleted for the last 275 amino acids of ORF2.

BD Living Colors Fluorescent Protein Vector (BD Biosciences Clontech) clones were used to visualize L1 proteins in living cells using fluorescent microscopy. Wild-type ORF2 was cloned by PCR methods downstream of enhanced GFP in the vector pEGFP-C3. No fluorescence was detected from this construct (pEGFP-C3-ORF2 WT) in living HeLa or 143B cells. An NcoI–NcoI fragment from pEGFP-C3-ORF2 WT, including EGFP and most of ORF2, was swapped into pMINI-FLAG-ORF2 WT to create pMINI-GFP-ORF2 WT. A BglII–EcoNI fragment from pMINI FLAG-ORF2 H230A/D702Y was swapped to create pMINI-GFP-ORF2 H230A/D702Y.

In a similar manner, ORF2 was cloned upstream of EGFP in the vector pEGFP-NI to create pEGFP-NI ORF2 WT. Again, ORF2p was not expressed at detectable levels. An AflII–NotI fragment from pEGFP-NI-ORF2 WT was introduced to pMINI-FLAG-ORF2 WT to create pMINI-FLAG-ORF2 WT-GFP, and finally pMINI-FLAG-ORF2 H230A/D702Y-GFP was produced by introducing the BglII–EcoNI fragment from pMINI-FLAG-ORF2 H230A/D702Y. pMINI-ORF1-RFP contains ORF1 with a C-terminal red fluorescent protein tag obtained from the vector pDsRed1-N1.

PCR techniques were used to insert an NcoI site at the 5’ end, and a T7-tag with AAGG-residue linker and terminal SalI site

Figure 4. Expression of L1 proteins from the following constructs: (A) pEGFP-C3-ORF1 (N-terminal GFP-tagged ORF1) in living HeLa cells; (B–G) pMINI-ORF1-T7 (C-terminal T7-tagged ORF1) in the MVA/T7RP system, examined by immunofluorescence of fixed 143B cells. ORF1p is shown present (B) and absent (E) from nucleoli. (H–M) Coexpression of ORF1 and ORF2 from pMINI ORF1-T7-IRES-FLAG-ORF2 H230A/D702Y (a bicistronic construct with upstream T7 promoter and an IRES at the beginning of each ORF) by the MVA/T7RP system and examined by immunofluorescence of fixed 143B cells. ORF1p is shown present (H) and excluded (K) from nucleoli in the presence of ORF2p (I and L).
at the 3’ end of ORF1 of retrotransposition-competent L1-RP (52) allowing cloning of pMINI-ORF1-T7. To create the bicistronic construct pMINI ORF1-T7-IRES-FLAG-ORF2 H230A/D702Y, ORF1 together with IRES and XbaI-A30 sequences (Fig. 1B) was extracted from pMINI-ORF1-T7, and cloned between the T7P and IRES of ORF2 in the constructs pMINI-FLAG-ORF2 WT and pMINI-FLAG-ORF2 H230A/D702Y.

Cell transfection and infection

Human umbilical cords were obtained from the Cardeza Foundation for Hematologic Research, Thomas Jefferson University. HUVEC cells were cultured as described (20). Human osteosarcoma 143B TK-cells (ATCC) were grown in high glucose DMEM medium with 10% FBS/glutamine/pen-strep.

Cell transfection and infection (MOI) of 10 in 1 ml OPTI-MEM (Invitrogen), followed after 40 min by plasmid transfection using the liposomal reagent FuGENE6 (Roche). Cells were harvested and transfected as described (21). 143B cells were seeded in six-well dishes and infected with MVA/T7RP at an multiplicity of infection (MOI) of 10 in 1 ml OPTI-MEM (Invitrogen), followed after 40 min by plasmid transfection using the liposomal reagent FuGENE6 (Roche). Cells were harvested in PBS/5 mM EDTA, washed once and infected with MVA/T7RP at a multiplicity of infection (MOI) of 10 in 1 ml OPTI-MEM. Cells were fixed, immunostained and assayed for sensitivity to 100 nM leptomycinB (LB, Sigma). Incubation conditions were: (i) 3 h with LB added to complete media 14 h post-transfection; (ii) overnight with LB in complete media; and (iii) overnight with LB in OPTI-MEM. Cells were fixed, immunostained and examined for increased nuclear localization.

The following fragments containing potential NESs were fused with a 3-alanine linker to the C-terminus of GFP in vector pEGFP-C3 or the N-terminus of GFP in pEGFP-N1 (Clontech): (i) ORF1 82–100 (LKELMEL); (ii) ORF2 232–258 (IKLELRIKNL); and (iii) ORF2 1160–1168 (LRDLELEI). Localization of GFP was examined in living 143B cells.

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