THE RNA TRANSPORT ELEMENT OF THE MURINE MosD RETROTRANSPOSON REQUIRES LONG-RANGE INTRAMOLECULAR INTERACTIONS FOR FUNCTION*

Michal Legiewicz1, Andrei S. Zolotukhin2, Guy R. Pilkington2, Katarzyna J. Purzycka1, Michelle Mitchell1, Hiroaki Uranishi3, Jenifer Bear2, George N. Pavlakis3, Stuart F.J. Le Grice1* and Barbara K. Felber2*

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Address correspondence to: Stuart F.J. Le Grice, Ph.D. and Barbara K. Felber, Ph.D., P.O. Box B, Building 535, Room 206, Frederick, Maryland 21702-1201. Fax: 301-846-7146; E-mail: legrices@mail.nih.gov and felberb@mail.nih.gov

Retrovirus replication requires specialized transport mechanisms to export genomic mRNA from the nucleus to the cytoplasm of the infected cell. This regulation is mediated by a combination of viral and/or cellular factors that interact with cis-acting RNA export elements linking the viral RNA to the cellular CRM1 or NXF1 nuclear export pathways. Endogenous type D murine LTR-retrotransposons (MosD) were reported to contain an RNA export element located upstream of the 3′ LTR. Although functionally equivalent, the MosD export element, termed MTE, is distinct from the other retroviral RNA export elements, such as the CTE of simian SRV/MPMV retroviruses and the RTE found in rodent IAP-LTR retrotransposons. We demonstrate here that the minimal RNA transport element (MTE) of MosD comprises multiple secondary structure elements that presumably serve as recognition signals for the cellular export machinery. We identified two classes of tertiary interactions, namely kissing loops and a pseudoknot. This work constitutes the first example of an RNA transport element requiring such structural motifs to mediate nuclear export.

Posttranscriptional control is essential for expression of cellular and viral mRNAs, involving complex interactions of mRNPs with transport receptors and components of the nuclear pore complex. Retroviruses depend on specialized transport mechanisms for nuclear export of full-length mRNA in their unspliced form, since this transcript encodes the gag-pol polyprotein and additionally serves as the genomic RNA packaged into progeny virions in the cytoplasm. For HIV and all lentiviruses, the human T-cell leukemia virus family, endogenous human retroviruses and mouse mammary tumor virus, transport of full-length mRNA depends on specific cis-acting RNA export signals that bind viral trans-acting factors and link the mRNA with the cellular CRM1 export receptor (1,2). In contrast, export and expression of the primary transcript of simple retroviruses and retroelements depends solely on cellular export machinery. Simian retrovirus (SRV) Mason Pfizer monkey virus (MPMV) contain the cis-acting CTE element (3,4) and some rodent LTR-Intracisternal A Particle (IAP) retroelements contain a CTE-like element designated the RNA Transport Element (RTE, (5,6)). These export elements represent interaction sites for cellular factors that provide a molecular link to the NXF1 export receptor (7-9). Inactivating the retroviral export machinery by removing the cis-acting RNA element or trans-acting protein factors results in nuclear retention of the primary transcript, and consequently impaired virus or retroelement production.

Like LTR-IAP, the murine Long Terminal Repeat (LTR) retrotransposon type D (MosD) and its derivative the Early Transposon (ETn) are mobile elements that share ancestry and genomic organization with retroviruses and contribute strongly to the ongoing spontaneous mutagenesis in mice (10-12). Analogous to other retroviruses and retroelements, the MosD LTR-retrotransposon contains an RNA element (termed here the MosD Transport Element, or MTE) that promotes nuclear export (11) using the cellular export machinery. This element is functionally equivalent to CTE and RTE but lacks sequence homology with known RNA export elements. It may therefore use
novel cellular factors or combinations of known factors to facilitate export. As a first step in understanding MTE-mediated RNA transport, we have undertaken a detailed structure-function study, combining in vitro chemical probing with site-directed mutagenesis and evaluation of RNA transport activity in vivo. Collectively, our studies highlight two classes of tertiary interactions that are essential for biological function, likely mediating recognition of MTE by trans-acting host factors.

**Experimental Procedures**

**Cell Culture and Transfection.** Hela-derived HLTat cells produce HIV-1 tat protein that is essential to activate expression from the HIV LTR promoter used in the pNLgag plasmid (8). Cells were transiently transfected with 200 ng of the gag reporter plasmid together with 200 ng of the GFP expression plasmid FRED25 via Superfect (Qiagen). Gag p24 was measured from supernatants and cell extracts 2-3 days posttransfection using the HIV p24 gag antigen capture assay (Zeptometrix) and GFP fluorescence was measured from cell extracts as described (8).

**In vitro RNA Synthesis and Folding.** MTE RNAs were purified from denaturing 4% polyacrylamide/8M urea gels. 20 pmoles of RNA were incubated at 95°C for 3 min in 20 µl of buffer containing 10 mM Tris pH 8.0, 100 mM KCl and 0.1 mM EDTA and placed on ice. The volume was adjusted to 150 µl in a final buffer of 40 mM Tris pH 8.0, 130 mM KCl, 4 mM MgCl2, 0.1 mM EDTA. Samples were incubated at 37°C for 15 min.

**SHAPE Analysis of MTE RNA.** Folded RNA was divided equally into two tubes (72 µl). 8 µl of 1M7 in DMSO (Sigma; anhydrous) or DMSO was added. The final 1M7 concentration was 2.5 mM for full length MTEs and 6 mM for truncated constructs. Samples were incubated for 2 min at 37°C, precipitated and resuspended in 10 µl TE (final concentration ~1 µM). Primer extension reactions were performed as described earlier (13), but ~5 pmoles of modified RNA were annealed with 5 pmoles of Cy5-end labeled reverse primer (IDT). Dideoxy sequencing markers were generated using unmodified RNA. cDNA extension products in deionized formamide with 0.5 µl of Standard Size 600 (Beckman) were analyzed by capillary electrophoresis (Beckman CEQ8000). The instrument setup was as stated (14).

Peak areas with single nucleotide resolution were integrated using the Lorentzian algorithm as implemented in CAFA0.41 (14). Intensities of integrated peaks were corrected for stochastic drop-off (15). Baselines were adjusted by dividing intensities by an average of five lowest intensities at invariant positions (identified manually). Intensities were normalized as described (16) and introduced into RNAstructure 4.6 (17). Tertiary interactions were introduced manually.

**aiSHAPE.** 2′-O-methyl-containing RNA oligonucleotides were purified from 15% denaturing polyacrylamide gels and stored in -20°C. The sequences of fully-modified 2′-O-methyl RNA oligonucleotides and LNA/DNA chimeras are provided in Supplementary Table 1. 2′-O-methyl-substituted oligonucleotides were added at a 2-fold excess before folding and chimeric LNA/DNA oligonucleotides after folding at a 10-fold excess. Samples were subsequently incubated at 55°C for 5 minutes and 37°C for 15 minutes prior to 1M7 treatment (see above). To quantify alterations induced by antisense oligonucleotides, raw data was processed as described above. aiSHAPE is defined as: (I - N) / (I + N) × 100, were ‘I’ defined 1M7 reactivity obtained in the presence of an interfering oligonucleotide and ‘N’ is the reactivity of native RNA.

**Native gel electrophoresis.** ~5 pmoles of [32P]-labeled MTE RNAs were folded as described for SHAPE experiments, in a final volume of 20 µl. Aliquots were loaded directly onto pre-cooled gels in 0.5 × Tris/Borate buffer containing 4 mM MgCl2. Fractionated RNAs were visualized by phosphorimaging on a Typhoon TRIO+ Imager (Molecular Dynamics).

**RESULTS**

The MTE secondary structure predicts long range interactions. Based on previous mapping studies (11), a 412 nt RNA, spanning nt 6254-6665 of MusD retrotransposon, was examined by SHAPE (16,18-20) (Fig. 1 and supplemental Fig. S1). Low chemical reactivity was noted for nucleotides of loop 3 (L3, nt C81-A89) and internal loop 8 (IL8, nt A180-U189). In addition, nt A271-A276, which were predicted to constitute
an apical loop of a short hairpin (Fig. 1 inset), displayed low 1M7 reactivity. We also expected that if nucleotides U222-C228 and G245-U250 were involved in forming an unstable stem extending S12 (Fig. 1 inset) they would be more reactive. Therefore we considered the notion that the MTE contained one or more tertiary interactions. Closer inspection of L3 and IL8 sequences revealed complementarity over 8 consecutive nucleotides, suggesting a kissing interaction. In contrast, chemical probing indicated that MTE nts G217-G312 assumed a more complex pseudoknot structure. The flanking positions of the terminal loop L12, designated here as IIA and IIA, interact with a central tract (nt G268-C279) of an internal loop (nt C263-U285) that interrupts stems S13 and S14 Consequently, two new stems, designated IIA/IIB (nt U222-C228 and G268-A274) and IIA/IIB (nt G245-C249 and G275-C279) and additional two loops, J13II and J13II, are formed (Fig. 1).

Mutagenesis of the MTE L3/IL8 kissing loops. To verify the L3/IL8 kissing interaction, we determined the SHAPE profiles for disruptive (MTE m2 and m4) and complementary MTE mutations (MTE m3 and m5, Fig. 2 and supplemental Fig. S2). Since all mutants retained their global MTE structure, the data of Fig. 2 and supplemental Fig. S2 illustrates only the region encompassing the kissing-loops. L3 nucleotide identity of MTE m2 was retained, while 8 nucleotides in the complementary IL8 were altered (Fig. 2A). Enhanced 1M7 reactivity of L3 and IL8 nucleotides indicated disruption of the kissing loops. Mutant MTE m3 (Fig. 2B) retained the equivalent IL8 alterations, while complementary mutations were introduced into L3. Surprisingly, although L3/IL8 complementarity was restored, the SHAPE profile of MTE m3 (supplemental Fig. S2B) resembled that of MTE m2, indicating the absence of a kissing interaction. As Fig. 2B shows, the number of single-stranded nucleotides comprising L3 has been reduced from nine to five – as a consequence of extending the adjacent stem 3 by two bp (nt C81-G89 and C82-G88). Apparently, these two newly formed G:C pairs are incorporated into a more stable stem, S3, reducing the number of accessible L3 nucleotides for pairing with IL8. Mutant MTE m4 (Fig. 2C) contained a wild type L3 sequence and a 3-nucleotide (C186-C188) alteration in IL8 to destabilize the kissing interaction. SHAPE data supported disruption of the kissing loops which was accompanied by enhanced reactivity of L3 nucleotides (supplemental Fig. S2C). A structural model for MTE m4 predicted local rearrangement of a region spanning the native IL8 (Fig. 2C), which partially explains its low reactivity (supplemental Fig. S2C). It has been previously shown that certain C residues predicted to constitute loops may display lower reactivity towards 1M7 (21). Additionally, it has been observed (22) that stretches of poly-C can form single stranded helical-like structures, which could account for lower reactivity in this region and several C-rich regions shown in Fig. 2. Finally, a complementary 3-nt change in L3 of MTE m5 produced SHAPE reactivity profile equivalent to that of the wild type MTE (supplemental Fig. S2D), suggesting restoration of the kissing interaction (Fig. 2D). Collectively, our mutagenesis analysis supported the notion of a long-range interaction between MTE L3 and IL8.

Examining tertiary interactions by antisense-interfered (ai) SHAPE. In order to more fully characterize the MTE pseudoknot, we modified the SHAPE strategy to address tertiary interactions. We reasoned that displacing one strand of an RNA duplex by hybridizing an antisense oligonucleotide would disrupt long-range interactions and be characterized by enhanced 1M7 reactivity of the displaced nucleotides. We designated our approach “antisense-interfered” or ai-SHAPE. In order to verify this strategy experimentally, we elected to interrupt the L3/IL8 kissing interaction by hybridizing the chimeric LNA/DNA octanucleotide 1B (supplemental Table S1) to IL8 sequence U182 – U189. As indicated in Fig. S3A, this elicited enhanced 1M7 reactivity of L3 nucleotides C83, U84 and A86 – A89, together with minor “off-target” effects at neighboring nucleotides A77 and U74. The specificity of the 1B-IL8 interaction is illustrated in the 1M7 reactivity profile of Fig. S3B, which shows little additional perturbation of RNA structure between MTE nts U60 and C170.

ai-SHAPE interrogation of the MTE pseudoknot. Having verified our ai-SHAPE approach experimentally with the L3/IL8 kissing interaction, a series of antisense oligonucleotides (supplemental Table S1) were hybridized to
several regions of the MTE pseudoknot, the results of which are summarized in Figs. 3A-E. 1M7 reactivity profiles for the entire 412 nt MTE indicated that all structural changes were restricted to the pseudoknot sub-domain. Oligonucleotide 3A, which is complementary to strand IIIA nucleotides G245-C249 (Fig. 3A), increased 1M7 reactivity (i.e., displacement) of strand IIIB nucleotides A274–C278. Although the effect was less pronounced, hybridizing oligonucleotide 3B, which complements strand IIIA nucleotides G275–C279, induced increased reactivity of strand IIIA nucleotides C247 and U248 (Fig. 3B). Oligonucleotide 6 extends complementarity beyond strand IIIB to include single-stranded nucleotides G275–G284 (Fig. 3C), and was designed to further destabilize the IIIA/IIIB duplex. Fig. 3C illustrates that this oligonucleotide elicited enhanced 1M7 reactivity of strand IIIA nucleotides G246 and C247, in addition to C244. Loop nucleotides A238-C244 of the proposed pseudoknot do not appear to contribute to tertiary interactions. However, if our structure prediction was correct, reducing their flexibility by hybridizing oligonucleotide 4 should affect the stability of nearby stem IIIA/IIIB. As shown in Fig. 3D, hybridizing oligonucleotide 4 resulted in increased 1M7 sensitivity of IIIB nt G275, A276 and G277 and strand IIIA nt C247 and U248.

Finally, oligonucleotide 2B complementary to nt U270 - C279, was designed to simultaneously interrogate stems IIIA/IIIB and IIIA/IIIB. Hybridization of 2B elicited enhanced 1M7 reactivity of strand IIA nt G223, G224, U225 and A226, consistent with disruption of the duplex (Fig. 3E). The effects of 2B on stem IIIA/IIIB were confined to C247, which, although somewhat restricted, is in keeping with data of Figs. 3B-D. We also noticed that oligonucleotide 2B induced several “off-site” effects. Enhanced 1M7 reactivity from C263–A266 might reflect proximity to stem IIIA/IIIB, while reactivity of G312–U315, G256 and C306 may represent spatial changes induced by the new 10-bp duplex resulting from hybridization of 2B to this compact sub-domain. Despite these minor discrepancies, the data of Fig. 3 lend credence to a complex series of tertiary interactions within the MTE pseudoknot.

Site-directed mutagenesis of the MTE pseudoknot. aiSHAPE was further complemented by assessing the effects of disruptive mutations on MTE pseudoknot architecture (Fig. 4). Mutant m7 preserved the sequence of stem IIIA/IIIB (cf Fig. 1 inset), while strand IIA nt U222–C228 were altered from U-G-G-U-A-C-C to A-A-G-A-G-C-C (mutagenized positions are indicated in bold and underlined), creating a 4-base mismatch. Fig. 4A and supplemental Fig. S4A indicate that this alteration was severely destabilizing, creating an extended stem IIIA/IIIA and a short stem-loop comprising IIIB/IIIB nucleotides. Nucleotides within the newly formed loop (A271 - A276) are highly reactive. Remarkably, this structural model closely resembles the predicted wild type MTE structure of Fig. 1, inset. It is important to recall that the wild type MTE and m7 displayed different SHAPE profile for nts G217 – G312. Interestingly, reduced 1M7 sensitivity of loop nucleotides C240, C241, U243 and C244 was observed. We propose that this homopolymeric tract is involved in the single stranded helical-like structure formation. Destabilizing the pseudoknot in MTE m7, and hence the constraining force caused by such a structure, may result in altered flexibility of L12 loop nucleotides and formation of a helical-like structure involving nt C240-C244 (Fig. 4A). In contrast, the presence of a pseudoknot may limit conformational freedom, which in this case is expressed as higher 1M7 reactivity of those residues in the wild type MTE (Fig. 1 inset).

The sequence of strand IIIB nucleotides G268 – A274 was altered from G-G-U-A-C-C-A to G-G-U-A-G-G-A in MTE mutant m8, and resulted in increased 1M7 reactivity of the counterpart strand IIA (Fig. 4B and supplemental Fig. S4B). Stem IIIA/IIIB was preserved, but now constituted part of an extended secondary structure that included strand IIIB nucleotides. At the same time, strand IIA nucleotides re-organized into loop nucleotides of a short stem-loop. Mutant MTE m6 was designed to interrogate the role of stem IIIA/IIIB by retaining the identity of stem IIA/IIIB while converting strand IIIA nucleotides G245 – C249 G-G-C-U-C to C-U-G-C-G. As would be predicted, Fig. 4C and supplemental Fig. S4C indicate that completely altering IIIA strand sequence disrupts the IIIA/IIIB duplex. However, based on SHAPE-constrained folding and the 1M7 reactivity pattern, the pseudoknot comprising stem IIIA/IIIB was preserved.

Finally, we reasoned that if the MTE pseudoknot represented an independently-folded,
compact unit, its architecture would be preserved in the isolated subdomain, spanning nt A204 – U320. Fig. 5, supplemental Fig. S5A and supplemental Fig. S5B show that important structural features of the wild type pseudoknot are indeed retained. Equally encouraging was our observation that the SHAPE signature of mutant m6, which is maintained only by the IIA/IIB interaction, corresponded with its structure in the context of the full-length element. In addition, MTE m6 migrates faster by native PAGE than the wild type construct, suggesting a highly compact structure (supplemental Fig. S5C).

**Long-range interactions are required for MTE biological function.** Using a previously described HIV-1 based gag reporter assay (6,9,23), we examined the effects of both kissing loop and pseudoknot mutations on MTE-mediated RNA transport (Figs. 6A and 6B, respectively). Gag expression was fully abrogated in kissing loop mutant MTE m2, whose altered IL8 sequence eliminated the kissing interaction (Fig. 2A). Likewise, MTE m3, which also failed to promote a kissing interaction (Fig. 2B), showed severely impaired gag expression levels, consistent with the notion that reducing the number of base pairs participating in the kissing loops decreased their stability. Mutant MTE m4, whose 3-nt mutation in IL8 destroyed the kissing loops, was also inactive (Fig. 2C). However, in keeping with structural probing data, elevated Gag levels were found for mutant MTE m5, whose L3 nucleotide changes restored the 8 bp L3/IL8 complementarity (Fig. 2D). The observation that Gag levels exceeded wild type may be in part due to the additional stability the three G:C pairs impart on the kissing interaction. Thus, although the precise role of this tertiary interaction remains to be elucidated, maintaining its architecture is critical for biological function.

Fig. 6B illustrates the effect of pseudoknot mutations m6–m8 on MTE function. Mutant m7, which disrupted both IIA/IIB and IIIA/IIIB pseudoknots, reduced gag levels to those of the control plasmid. Similarly, mutant m8, which retains IIIA/IIIB base pairing as simply a secondary interaction without pseudoknot formation, was also inactive. In contrast, MTE mutant m6, which retains a pseudoknot comprising stem IIA/IIB, supported gag expression at a level ~30% of the wild type MTE. In combination with chemical probing, *in vivo* data suggests that both the IIA/IIB and IIIA/IIIB interactions are required for full MTE activity.

**DISCUSSION**

While it has been established that the 3′ untranslated region of the MusD RNA genome harbors a cis-acting element necessary for nuclear mRNA export (11), the structure of this ~400 nt RNA and how this contributes to efficient nuclear export remain to be deciphered. As a first step in this direction, we determined the entire MTE RNA structure by chemical footprinting. Herein, we present evidence for tertiary interactions within its 5′ and 3′ domains, with the former harboring a kissing loop and the latter a dual pseudoknot. SHAPE data for both tertiary elements have been complemented by functional studies that demonstrated their necessity for MTE activity. Originally defined as an intermolecular interaction important for HIV-1 RNA genome dimerization (24), kissing loops have also been identified in the genomes of hepatitis C virus (25), chrysanthemum chlorotic mottle viroid (26) and a group C enterovirus (27). Furthermore, although a role for the MTE pseudoknot in RNA transport remains to be established, equivalent elements are associated with translational control via internal ribosome entry sites (28,29), ribosomal frameshifting (30) and tRNA mimicry (31,32). Our work provides the first example of this kind of structural motif involved in nuclear export. Although more than 100 MusD sequences have been identified in the mouse genome (10), only nine of these have uninterrupted open reading frames, of which three are autonomous for transposition. For these three elements, nucleotides constituting the kissing loops and pseudoknot stems IIA/IIB and IIIA/IIIB retain complementarity, suggesting evolutionary conservation.

Our demonstration that structural changes arising from inactivating mutations, and compensatory changes restoring MTE function, are limited to these two tertiary interactions indirectly suggests the kissing loop and pseudoknot function as independent structural/regulatory elements. Furthermore, since MTE kissing loop mutant m5 supports wild type nuclear export levels by retaining complementarity with an altered nucleotide sequence, the notion
that the $L3/IL8$ interaction provides sequence-specific recognition for host export factors also seems less likely. Alternative possibilities would be that the kissing interaction is a critical recognition element, or that it maintains the spatial arrangement of adjacent recognition sites for host proteins. Supporting this notion, kissing loops often adopt distorted A-form geometry that induces a bend in the complex, providing a recognition site for ligands (33). The same argument holds for the pseudoknot, where the stem $IHA/IIB$ interaction alone suffices to maintain general features of the pseudoknot architecture and preserve biological activity. Defining the roles of host nuclear export factors is essential to get a fuller understanding of MTE function. The export of other retroviral RNA export elements (CTE and RTE) was reported to include NXF1 (9,36), the RNA binding motif protein 15 [RBM15] (8,9,34), and OTT3 [also referred to as RBM15B] (9,35). The contribution of these and other cellular factors involved in MTE RNA transport is under investigation. Given the complexity of the MTE reported here, and the fact that thermodynamic stability of pseudoknots control ribosomal frameshifting (30), it would not be unreasonable that the interaction with cellular factors triggers further conformational changes to promote passage of the nucleoprotein complex through the nuclear pore.

A recurring theme in understanding the mechanism and function of retroviral RNA export elements is that they span ~200-400 nt and are highly structured. For example, the HIV RRE comprises several stem-loops that contribute to RRE function in addition to a binding site of the Rev protein (13,23,37-41). Likewise, the CTEs of simian Type D retroviruses and rodent LTR-retrotransposons (5,42-44) contain four conserved sequence motifs that define binding sites of the cellular NXF1 export receptor, arranged in two mirror-symmetrical pairs that form the internal loops of an extended hairpin loop (3,5,7,46). Lastly, the RTEs of murine IAP LTR-retrotransposons (6,47,48) are more complex, comprising four essential stem-loops (49). The complexity of the $MusD$ MTE provides another example to this series of highly structured elements. In contrast to other known RNA export elements CTE, RTE, and RRE, the MTE provides the first example of an element whose function is mediated by long-range, intramolecular interactions. Our detailed examination of the MTE structure is an important step towards unraveling mechanisms that govern this critical step in posttranscriptional regulation. An in-depth analysis of MTE function will thus add to our understanding of mechanisms that mediate posttranscriptional control of cellular transcripts.

REFERENCES


**FOOTNOTES**

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The abbreviation used are: CRM1, chromosome region maintenance; NXF1, nuclear export factor 1; MusD, murine LTR-retrotransposon type D; MTE, minimal RNA transport element; nt, nucleotide; SHAPE, Selective 2′-Hydroxyl Acylation analyzed by Primer Extension; 1M7, 1-methyl-7-nitroisatoic anhydride; MTE, MusD retroelement transport element; RTE, RNA transport element; CTE, constitutive transport element; RRE, rev-responsive element

**FIGURE LEGENDS**

Fig. 1. MTE secondary structure and architecture of tertiary interactions. 1M7 reactivity (SHAPE output data) at each nt position is color-coded according to the normalized scale. Tertiary interactions include base pairing between loops L3 and IL8 (kissing loops), and a dual pseudoknot seen as a cross-over of regions: flanking positions of a loop L12 – IIA (U222-C228) and IIIA (G245-C249) with a continuous nucleotide tract IIIB (G268-A274)-IIIB (G275-C279). Loops J13/II (C263-A267) and J14/IIIB (A280-U285) join IIIB/IIIB tract with stems S13 and S14, respectively. Arrows in structure indicate 5′-3′ RNA strand direction. S = stem, SL = stem-loop, IL = internal loop, includes single-stranded regions from the counter strand, J = junction. Boundaries of a minimal functional MTE RNA are marked as position 1 and 412. Inset; predicted secondary structure of the pseudoknot sub-domain with introduced interactions IIA/IIIB and IIIA/IIIB represented by purple and green lines, respectively.

Fig. 2. *In vitro* chemical probing of MTE variants with altered kissing loops. Arrows indicate site-directed mutagenesis in the direction native to mutant sequence. Color-coding of 1M7 reactivity is as in the legend to Fig. 1. A-C, MTE variants (m2-m4) with disrupted kissing loops; D, MTE with compensatory mutations to variant m4 that restore kissing loops.

Fig. 3. Structural responses of MTE pseudoknot domain to antisense oligomers monitored by aiSHAPE. Influence of antisense oligonucleotides 3A (A), 3B (B), 6 (C), 4 (D) or 2B (E) on SHAPE profiles. Sequences of the interfering oligonucleotides are provided within each structure in orange font; uppercase in 3A and 3B oligomers indicates an LNA and lower case – a DNA position. Nucleotide positions exhibiting increased 1M7 reactivity in the presence of given oligomer are depicted by orange squares. Plots of 1M7 reactivities of native and antisense-interfered MTE RNA are shown in the accompanying inserts.
Fig. 4. Site-directed mutagenesis of the MTE pseudoknot domain. Secondary structure models of the pseudoknot domains of MTE mutants m7 (A), m8 (B) and m6 (C), based on SHAPE-constrained folding. Arrows indicate site-directed mutagenesis in the direction native to mutant sequence. Color-coding of 1M7 reactivity is as in the legend to Fig. 1. Retained tertiary interactions are represented by purple lines.

Fig. 5. Analysis of isolated pseudoknots of MTE wt and Mutant m6. Difference plots of SHAPE signature between full-length MTEs (red) and truncated constructs (blue). Numbering correspond to the full-length MTE RNA. Regions involved in the pseudoknot formation are marked with dashed boxes in the plot. Significant differences in the 1M7 reactivity between isolated pseudoknots and pseudoknots in the sequence context of the full length MTEs are indicated by arrows and do not introduce changes into structural models of RNA pseudoknots.

Fig. 6. In vivo analysis of MTE mutants reveals functional importance of RNA tertiary interactions. Point mutations were introduced into the critical residues of the predicted kissing loops (A) or pseudoknot (B) and cloned into the pNLgag reporter construct. Human HeLa-derived cells were transfected with 200 ng of the respective constructs and 200 ng of the GFP plasmid as internal transfection control. Total Gag production was measured by quantitative p24<sub>ag</sub> ELISA and presented as fold increase relative to the empty NLgag plasmid. Mean values of a representative experiment performed with triplicate plates are depicted and the values measured from the internal control GFP (firefly units, ffu) are indicated along with standard deviations. Nucleotide alterations are indicated in red, and “+” and “-“ denote whether in vitro chemical probing detected a tertiary interaction within MTE.
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### Figure 6.

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