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Identification of a Novel Posttranscriptional Regulatory Element by Using a *rev*- and RRE-Mutated Human Immunodeficiency Virus Type 1 DNA Proviral Clone as a Molecular Trap

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Human immunodeficiency virus (HIV) and all other lentiviruses utilize the essential viral protein Rev, which binds to RRE RNA, to export their unspliced and partially spliced mRNAs from the nucleus. We used a *rev*- and RRE-defective HIV type 1 (HIV-1) molecular clone in complementation experiments to establish a method for the rapid isolation of posttranscriptional regulatory elements from the mammalian genome by selecting for rescue of virus replication. Viruses rescued by this method contained a novel element with homology to rodent intracisternal A-particle (IAP) retroelements. A functional element was contained within a 247-nucleotide fragment named RNA transport element (RTE), which was able to promote replication of the Rev- and RRE-defective HIV-1 in both human lymphoid cell lines and primary lymphocytes, demonstrating its potent posttranscriptional function. RTE was functional in many cell types, indicating that the cellular factors that recognize RTE are widely expressed and evolutionarily conserved. RTE also promoted RNA export from *Xenopus* oocyte nuclei. RTE-mediated RNA transport was CRM1 independent, and RTE did not show high affinity for binding to mRNA export factor TAP/NXF1. Since CRM1 and TAP/NXF1 are critical export receptors associated with the two recognized mRNA export pathways, these results suggest that RTE functions via a distinct export mechanism. Taken together, our results identify a novel posttranscriptional control element that uses a conserved cellular export mechanism.

The study of retroviral mRNA expression has provided some important insights for the understanding of nucleocytoplasmic export and posttranscriptional regulation in mammalian cells. The process of mRNA splicing and transport is tightly controlled in retroviruses to ensure that both spliced and unspliced mRNAs are produced and transported to polysomes at the appropriate proportions. These pathways are regulated at the posttranscriptional level by *cis*-acting elements on the viral RNA and by cellular and viral proteins (for reviews see references 12, 25, 33, 46, 55, 60, and 64). The Rev-responsive element (RRE) of HIV-1 was the first to be identified as a unique RNA element within the *env* coding region of HIV-1. RRE binds the essential protein Rev and promotes the nuclear export, stability, and expression of all viral mRNAs containing RRE. It was subsequently found that all lentiviruses, some oncoretroviruses (for reviews see above), and the type D and the avian leukosis retroviruses have *cis*-acting RNA elements with analogous function (6, 49, 52, 62, 74). These elements have been shown to mediate RNA export, and some elements can be exchanged among the different viruses (6, 47, 50, 68, 74). Functionally related posttranscriptional regulatory elements have also been identified in hepatitis B virus (30), woodchuck hepatitis virus (10), and herpes simplex virus (44). Detailed analyses of some of these elements have shown that they bind different factors and direct RNA to different cellular export pathways, indicating distinct mechanisms of function (51, 52, 54, 56, 73). Thus, RRE-Rev of HIV-1 utilizes the CRM1/exportin-1 pathway (15, 16, 48). The constitutive transport element (CTE) of type D retroviruses utilizes the TAP/NXF1 export factor (21), which also represents a conserved mRNA export pathway (31, 36, 53, 56, 58, 63). Since cellular factors interact with the posttranscriptional control elements such as the CTE, it was hypothesized that similar elements may also be involved in the posttranscriptional regulation of cellular genes. Some evidence that cellular equivalents of the CTE exist in the mammalian genome has been reported for the mouse histone H2a gene (28, 29), although its mechanism of function is unclear.

We therefore designed a strategy to identify additional *cis*-acting RNA elements participating in nucleocytoplasmic transport of mRNAs. A molecular clone of HIV-1 with multiple mutations inactivating both *rev* and RRE, but not affecting the overlapping open reading frames for *tat* and *env*, was generated. We then inserted random fragments of genomic mouse DNA into this replication-defective molecular clone and selected for replicating virus in human cells. This powerful selection resulted in the identification of a novel posttranscriptional control element named RTE, which allows for efficient production and continuous propagation of the virus.

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MATERIALS AND METHODS

Trap system. pNL43Rev$^-$R$^-$ was generated by replacing the *Eco*RI-*Bam*HI fragment of pNL4–3 (1) with the *Eco*RI-*Bam*HI fragment from pNL43Rev$^-$R$^-$S (66, 74). The resulting plasmid, pNL43Rev$^-$R$^-$, contains the previously described mutations in *rev* and RRE (74) and has a unique *Xho*I site in *nef*. pSRV-1 DNA (65) and a mixture of mouse genomic DNA were partially digested with *Alu*I and *Rsa*I, respectively, at 37°C for 15 min. Digestion was terminated by heating at 70°C for 10 min. After fractionation by agarose gel electrophoresis, the mixture of 300- to 1,000-bp DNA fragments was isolated, purified, and cloned into the blunted *Xho*I site of pNL43Rev$^-$R$^-$. All fragments shown in Fig. 3A and B were cloned similarly into the *Xho*I site of pNL43Rev$^-$R$^-$. Fragments 3A, 3B, and 3C and fragments 30B and 30C were derived from clones 3 and 30, respectively. Individual clones containing insert 3B in sense (3Bs; clone 1) and antisense (3Bas; clone 11) orientations were used in several experiments. Fragment M1 was derived from 3B. The insertions in all molecular clones were verified by sequence analysis.

Virus stocks were generated after transfection of human transformed embryonic kidney cell line 293 (18) with the ligation mixtures or molecular clones. One day after transfection, the cells were washed and cocultivated with 2×10^6 Jurkat cells in 5 ml of fresh medium. Supernatants were collected, filtered through 0.45-μm-pore-size Millipore filters, and stored at −80°C. For cell-free infections, Jurkat cells (4×10^6) or phytohemagglutinin-stimulated peripheral blood mononuclear cells PBMCs (10^7) were washed once with phosphate-buffered saline (PBS) and infected with the virus supernatant in a final volume of 2 ml. The amount of input virus was adjusted for each experiment according to the HIV-1 $p24^{gag}$ concentration in the supernatants. After incubation for 4 h at 37°C, the PBMCs were washed twice with PBS and were cultured in RPMI medium supplemented with 10% fetal calf serum and 5 U of recombinant interleukin 2/ml. Jurkat cultures were split and fed with fresh medium twice a week. The cultures were monitored using either a commercial or an in-house (obtained from L. Arthur, SAIC-National Cancer Institute) $p24^{gag}$ antigen capture assay (74).

For long-term passage of virus, 1 ml of virus stock generated from 293 cells transfected with pNL43Rev$^-$R$^-$ containing fragment 3B was used to infect 2×10^6 to 3×10^6 Jurkat cells, which were cultured in 15 ml of medium. At days 4, 7, and 11, 12 ml of the culture medium was replaced with fresh medium and 1×10^6 to 2×10^6 fresh Jurkat cells were added. At day 14, 2 ml of the supernatant was transferred onto fresh Jurkat cells. For each round of cell-free infection, the Jurkat cells were cultured as described above and every 2 weeks the passaging was repeated. After passages 11, 18, and 42, DNA was isolated from the infected cells and subjected to PCR amplification using primers flanking the *Xho*I site. The PCR fragments were cloned into the TOPO vector (Invitrogen) and sequenced using T3 and T7 primers.

RNA export from *Xenopus* oocyte nuclei. RTEs spanning nucleotides (nt) 381 to 627 (M1) and nt 391 to 616 were cloned into the unique *Sac*II site of *Eco*RV-linearized plasmid pBSAd1 (37), in which the *Sau*3A site located within the second exon was replaced by unique *Eco*RV and *Mlu*I sites. The CTE-containing adenovirus precursor RNA was described previously (56). Radiolabeled RNA was prepared from 1 μg of the linearized plasmids using a MaxiScript kit (Ambion). Synthesis of all RNAs was primed with the G(5′)ppp(5′)G cap analog (Ambion). Transcription reaction mixtures were phenol extracted and purified on P30 gel filtration columns (Bio-Rad). The injection mixtures also contained U1ΔSm RNA and U6Δss RNA (34). Amounts of transcripts in mixtures were equilibrated according to specific activities. One injection contained approximately 2×10^4 cpm of pre-mRNA. The pelleted, dried transcripts were reconstituted in 20 mg of blue dextran/ml in water and used to inject *Xenopus* oocyte nuclei as described previously (34). Nuclear and cytoplasmic fractions were obtained after manual dissection. RNA was extracted from a pool of 10 oocytes after proteinase K digestion, and equivalents of one-half oocyte were loaded onto 10% polyacrylamide gels containing 7 M urea.

CAT expression analysis. The cells were transfected with 1 μg of plasmid DNA (purified by Qiagen columns) using the calcium phosphate coprecipitation technique (13, 19). Chloramphenicol acetyltransferase (CAT) expression vector pDM138-RTE contains the RTE (nt 391 to 616) inserted into the unique *Cla*I site of pDM138 (27); pDM128 contains the HIV-1 RRE (26); pDM138-CTE contains the CTE (51). pDM128 was cotransfected with 0.5 μg of *rev* expression vector pCMVRevsg25 (59). For CRM1 export inhibition, transfected monkey COS-7 cells were washed after 7 h, incubated in the presence of leptomycin B (LMB; a gift from B. Wolff) for 8 h, and harvested. Human 293 and quail QCL-3 cells (obtained from B. Cullen) were transfected and harvested 2 days later. The cell extracts were analyzed for CAT production (17).

Proviral DNA analysis. To prepare DNA from infected cultures, 10^6 Jurkat cells were digested for 18 h in 100 μl of lysis buffer (10 mM Tris-HCl [pH 8.3], 1 mM EDTA, 0.5% Tween 20, 0.5% NP-40) containing 400 μg of proteinase K/ml. The elements inserted into pNL43Rev$^-$R$^-$ were amplified from isolated DNA by 30 cycles of PCR using primer pair 3690 (nt 8304 to 8329 in HIV) plus 3805 (nt 8508 to 8538) and were subcloned into the pCRII TA cloning vector (Invitrogen). The numbering of the HIV-1 nucleotides follows the revised HXB2R sequence, where +1 corresponds to the mRNA start site (45, 57). The inserts were sequenced from both sides using primers 18209 (HIV nt 8800 to 8827) and 18330 (nt 8922 to 8948). Additional sets of primers derived from regions 758 to 788, 252 to 277, 411 to 431, and 521 to 540 of the cloned element were used for sequencing (the numbering of RTE follows the sequence of clone 1).

Specific fragments were amplified with the following primer pairs: Bio.86 (nt 1 to 11) and OSW.284 (nt 279 to 310) for fragment 3A, OSW.281 (nt 296 to 310) and OSW.282 (nt 674 to 688) for fragments 3B and 30B, and OSW.283 (nt 686 to 784) and Bio.89 (nt 1075 to 1086) or Bio.90 (nt 968 to 981) for fragment 3C or 30C, respectively. Sequences were obtained by the PCR-assisted fluorescent terminator method (ReadyReaction DyeDeoxy terminator cycle sequencing kit; PE Biosystems). Sequencing reactions were run on the Applied Biosystems model 373A DNA sequencing system. Sequencing data were analyzed by the Sequencher software, version 2.1.1, package (Gene Codes Corp.).

Computational analysis. Sequence homology searches were performed against the nonredundant nucleotide sequence database by using the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST) to identify homologous sequences. Repetitive sequences were identified using the World Wide Web-based RepeatMasker tool (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker). Alignments were generated by the Genetics Computer Group (GCG) program Pileup (9), using the sequences identified by the BLAST homology search. The evolutionary analysis of the aligned sequences was performed with the SplitsTree analysis tool (32).

In vitro RNA synthesis. The DNA templates for histone H4, simian retrovirus type 1 (SRV-1) CTE, and CTE mutant M36 RNAs were described previously (5, 21, 56). RTE (M1; nt 381 to 627) was inserted into *Bam*HI and *Xho*I sites of the Bluescript KS(+) plasmid, linearized with *Xho*I, and transcribed from the T7 promoter. BS is a 100-nt RNA produced from the in vitro transcription of the *Xho*I-digested Bluescript KS(+) plasmid using T7 RNA polymerase. Radioactive RNA probes were prepared as described previously (21). The unlabeled RNAs were synthesized using the Megascript kit (Ambion) and purified by phenol extraction and gel filtration on Sephadex G-50 columns.

RNA binding assays. For the binding reactions, ^{32}P-labeled RNA probes (10 nM for CTE or 20 nM for RTE) were incubated with 0.1 to 0.4 μg of purified glutathione *S*-transferase (GST)–TAP61–610 or GST–TAP61–372 proteins, respectively, in a final volume of 10 μl as described previously (21). For competition binding, various amounts of purified unlabeled RNAs were added to the reaction mixtures. The reactions were carried out for 10 min at room temperature in 15 mM HEPES, pH 7.7–50 mM KCl–0.2 mM EDTA–2 μg of heparin/ml in the presence of 200 or 25 mM NaCl for the CTE probe and the RTE probe, respectively. The complexes were separated on 1% agarose or 4% polyacrylamide gels and detected by autoradiography.

Nucleotide sequence accession numbers. The sequences of clones 1, 3, and 30 were deposited into the database under GenBank accession no. AF250998, AF250999, and AF251000, respectively.

RESULTS

The mouse genome contains posttranscriptional regulatory elements identified by using an HIV molecular trap. To identify novel posttranscriptional control elements, we used a new strategy which relies on the ability of heterologous RNA transport elements to complement a nonreplicating HIV-1 molecular clone. This clone, pNL43Rev$^-$R$^-$ (Fig. 1) contains multiple mutations that destroy both the RRE region and the *rev* open reading frame without affecting the overlapping *env* and *tat* open reading frames. NL43Rev$^-$R$^-$ (see Fig. 2), like the previously described NL43R$^-$ (74), does not produce any virus after transfection into HIV-susceptible cell lines. We previously showed that the presence of the SRV-1 CTE in NL43Rev$^-$R$^-$S resulted in production of stable virus that is unable to revert to wild type even after multiple passages (74).

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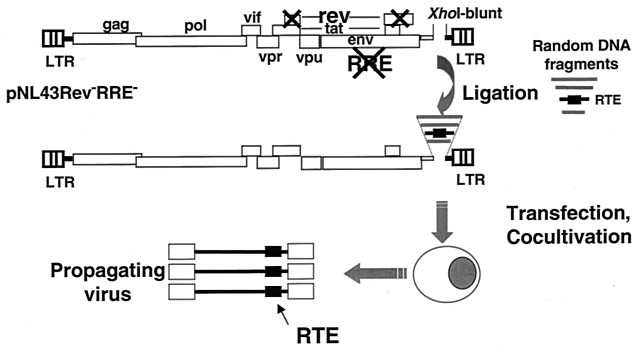


FIG. 1. The molecular trap for posttranscriptional control elements. The HIV-1 pNL43Rev$^-$R$^-$ molecular trap vector contains 37 nucleotide changes within the RRE and two point mutations within the first coding exon of *rev* that destroy the RRE and the *rev* open reading frame, respectively (74). These mutations do not affect the overlapping open reading frames for *tat* and *env*. Fragments of 300 to 1,000 bp obtained by digestion of heterologous DNAs were inserted into the filled-in *Xho*I site located in *nef*, and the ligations were directly transfected into human 293 cells. One-day posttransfection fresh Jurkat cells were added and culture supernatants were monitored for p24^{gag} production.

These properties of the molecular clone were essential for the establishment of valid conclusions about the expression and transmission of the mutated viruses in the present study.

To identify new posttranscriptional regulatory elements, we ligated DNA fragments of approximately 300 to 1,000 bp obtained after partial digestions of genomic DNA with two different enzymes (*Alu*I and *Rsa*I) into the *Xho*I-digested blunt-ended pNL43Rev$^-$R$^-$ molecular clone (Fig. 1). The *Xho*I site is located within the *nef* gene, which is not essential for virus growth in vitro. The ligation mixtures were directly transfected into human 293 cells, followed the next day by cocultivation with the CD4$^+$, HIV-permissive Jurkat cell line to allow for virus propagation, which was monitored using the p24^{gag} antigen capture assay. To further examine the potency of identified posttranscriptional elements, such virus stocks were routinely assayed in cell-free infection experiments using both Jurkat cells and human PBMCs.

As a proof of concept, serial dilutions of random fragments from a molecular clone of SRV-1 containing the previously identified CTE were mixed with Jurkat genomic DNA and subjected to the ligation-transfection-cocultivation protocol. Only the presence of the pSRV-1 fragments in the ligation resulted in virus propagation, while the control cultures containing only pNL43Rev$^-$R$^-$ remained negative (not shown). We concluded that the method was able to detect the presence of CTE sequences in a complex mixture of DNA fragments.

We next examined whether this approach was able to detect *cis*-acting RTEs contained within DNA isolated from mouse embryonic stem cells. Human 293 cells were transfected with the ligation mixture and monitored over time by measuring HIV p24^{gag} antigen in the supernatant. At day 14 of cocultivation, the culture was positive on the basis of syncytium formation and p24^{gag} antigen capture assays (Fig. 2A). As expected, the control culture infected with NL43Rev$^-$R$^-$S containing the SRV-1 CTE scored positive earlier, while mock-transfected cells and cells transfected with starting vector pNL43Rev$^-$R$^-$ did not show any virus propagation. We further showed that virus stocks prepared at day 24 (Fig. 2A) were also able to transmit virus to PBMCs upon cell-free infection (Fig. 2C; supernatants A and B). These results indicated that we rescued a strong posttranscriptional control element(s) able to promote high levels of infectious virus.

DNA from the cultures positive for virus propagation (Fig. 2A) was isolated at day 14 and analyzed by PCR to identify DNA fragments inserted into pNL43Rev$^-$R$^-$. Using primers flanking the *Xho*I cloning site, we detected multiple bands of approximately 700 to 1,200 bp (Fig. 2B, lane 3), indicating that the propagating virus contained functional posttranscriptional regulatory elements from the mouse genome.

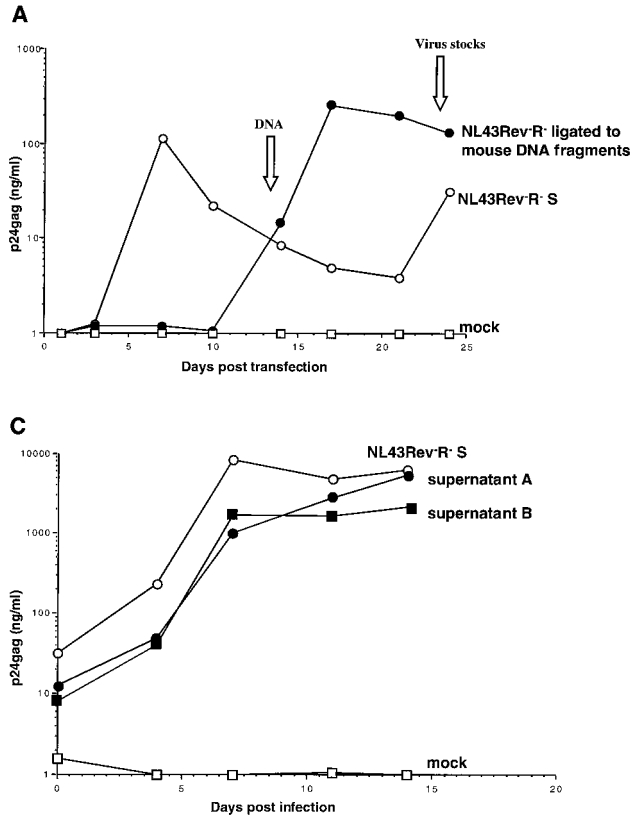
Characterization of the newly identified posttranscriptional control elements. To characterize the elements able to rescue viral production, the amplified DNA fragments shown in Fig. 2B (lane 3) were cloned and sequenced. Sequence analysis of 13 clones revealed that the inserts were similar in nucleotide sequence, as depicted in Fig. 3A. Several clones (clones 1, 3, 7, 9, 13, and 22) were 1,086 nt in length, whereas some others (clones 17, 25, 6, 12, 30, and 32) varied between 690 and 807 nt. Clones 17, 25, and 23 are identical to clone 1, except that they contain internal deletions. The overall lengths of the inserts are in excellent agreement with the experimentally detected fragment sizes shown in Fig. 2B, which also contain viral flanking regions.

Comparison of the ends of the inserted fragments demonstrated that at least two independent ligation events were selected during virus propagation. Clones 1, 3, 7, 9, 13, 22, 17, 23, and 25 have the same junctions to the virus DNA (Fig. 3A), indicating the same ligation event. Clones 6, 12, 30, and 32 form a second group having different junctions to viral DNA (Fig. 3A) and have identical stretches of 68 nt not found in the other group. Since the junctions to the *Xho*I-linearized HIV-1 molecular clone were different, we concluded that two distinct ligation events were propagated by virus rescue. Interestingly, only part of region B (see also below) is shared by all clones, and the region varies by only a few point mutations, except for clone 32, which has a 64-nt deletion.

To verify that the cloned elements were indeed able to replace Rev and RRE and rescue virus, selected fragments were ligated into *Xho*I-linearized pNL43Rev$^-$R$^-$ and were tested for their ability to produce infectious virus upon transfection into 293 cells and cocultivation in Jurkat cells. As summarized in Fig. 3A (see HIV rescue activity to the right), cultures transfected with ligations containing fragment 1 (1,086 nt), 3 (1,086 nt), 7 (1,086 nt), 17 (807 nt), 25 (800 nt), 6 (754 nt), 30 (754 nt), or 32 (690 nt) produced infectious virus, while a molecular clone containing clone 23 (135 nt) did not. Cell-free infection of Jurkat cells with virus stocks containing fragment 1, 3, 7, 17, 25, 6, 30, or 32 further confirmed that the viral particles produced were able to generate fully infectious viral progeny. These results verified that the virus rescue was indeed due to the presence of the cloned mouse fragments, which contain an RTE able to replace Rev and RRE.

The RTE is located within a 247-nt element present within a novel class of IAPs. Sequence analysis and homology searches showed that the group of the longest elements (1,086 nt; Fig. 3A) could be divided into three regions: A (nt 1 to 294), B (nt 295 to 687), and C (nt 688 to 1086). As depicted in Fig.

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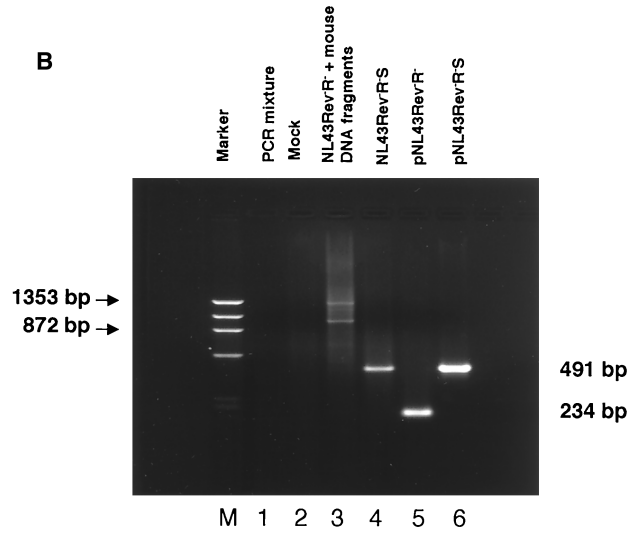


FIG. 2. Propagation of NL43Rev$^-$R$^-$ after ligation of mouse genomic DNA. (A) Fragments of mouse DNA were cloned into the HIV-1 pNL43Rev$^-$R$^-$ vector, and the ligation was transfected into 293 cells. One day later, the transfected cells were cocultivated with Jurkat cells. Cocultivations of the transfected cells containing mouse DNA fragments (solid circles) or the control NL43Rev$^-$R$^-$S virus containing the SRV-1 CTE (open circles) resulted in virus production. Mock-transfected cells (open squares) or cells transfected with the starting vector pNL43Rev$^-$R$^-$ (see also Fig. 4) never showed any virus production. (B) At day 14, DNA was isolated from the culture samples shown in panel A, amplified by PCR using primers flanking the *Xho*I site, cloned, and analyzed for the presence of inserts within the HIV *nef* open reading frame. Note that these fragments also contain viral flanking sequences. Lane 1, PCR mixture (no DNA); lane 2, Jurkat DNA from cocultivations of mock-transfected cells; lane 3, cells transfected by NL43Rev$^-$R$^-$ ligated to random fragments of mouse genomic DNA; lane 4, NL43Rev$^-$R$^-$S-transfected cells; lane 5, amplification of pNL43Rev$^-$R$^-$ plasmid DNA; lane 6, amplification of pNL43Rev$^-$R$^-$S plasmid DNA; lane M, ϕX *Hae*III-digested DNA used as a marker. (C) Analysis of rescued virus upon cell-free infection of human PBMCs. Viral stocks (supernatant A and supernatant B) were harvested at day 24 from two parallel Jurkat cell cultures as shown in Fig. 2A. NL43Rev$^-$R$^-$S virus served as a positive control. Equal amounts of virus (as determined by p24^{gag} antigen capture assays) were used for cell-free infection of PBMCs, and virus propagation was monitored over time by measuring p24^{gag} production.

3C, region A shows homology to the Line/L1 repeat element (nt 38 to 255) as determined by the RepeatMasker software program. Region B shows homology to intracisternal A-particle (IAP) retroelement-associated elements (nt 399 to 610). Region C shows 86% identity to CTE_{IAP} (nt 709 to 857), a previously identified functional CTE-related RNA transport element found within a retrotransposon located in the osteocalcin-related gene (GenBank accession no. U53820 [61]). The CTE_{IAP} homologous region is followed by a polypurine track element (nt 858 to 877) and a retroviral long terminal repeat (LTR) (RLTR10 [nt 879 to 1086]). Detailed inspection of the CTE sequence revealed that this element is shorter than the described CTE_{IAP} (61); it lacks one of the essential internal loops (binding site for TAP/NXF1) and has several point mutations within the internal stem structure. For these reasons, this CTE was predicted to be inactive, as verified experimentally (see below; Fig. 4B); hence it was named mutant CTE_{IAP} ($mCTE_{IAP}$). Taken together, the fragments rescued by the molecular trap show homology to IAP.

Based on the comparisons of all the active fragments (Fig. 3A), the middle region, B (spanning nt 295 to 687), was suggested to contain the active posttranscriptional control element RTE. This was further verified by the analysis of individual fragments spanning regions A, B, and C. Fragments 3A, 3B, and 3C from clone 3 and fragments 30B and 30C from clone 30 (Fig. 3B) were tested for their ability to mediate virus propagation. Figure 4A confirmed that only the presence of B fragments (3B and 30B) was able to rescue virus, whereas A and C fragments (such as 3A, 3C, and 30C) failed to do so. As predicted from the sequence analysis (see above), the $mCTE_{IAP}$ located within region C was not active due to the presence of multiple mutations. Recombinant viruses carrying fragments 3B and 30B propagated similarly to CTE-containing virus NL43Rev$^-$R$^-$S.

Fragment 3B was inserted in sense (3Bs) and antisense (3Bas) orientations into pNL43Rev$^-$R$^-$, and recombinant molecular clones were tested in cocultivation (data not shown) and cell-free virus transmission assays (Fig. 4B). Virus rescue required that the RTE be inserted in the sense orientation, since insertion of fragment 3B in the opposite transcriptional orientation (3Bas) failed to produce virus.

Comparison of the active fragments (Fig. 3A) revealed that the functional region could be further reduced to approximately 251 nt, since functional clone 32 contained an additional deletion (nt 313 to 378). In addition, we found that, upon cell-free passage, the virus containing fragment 3B ac-

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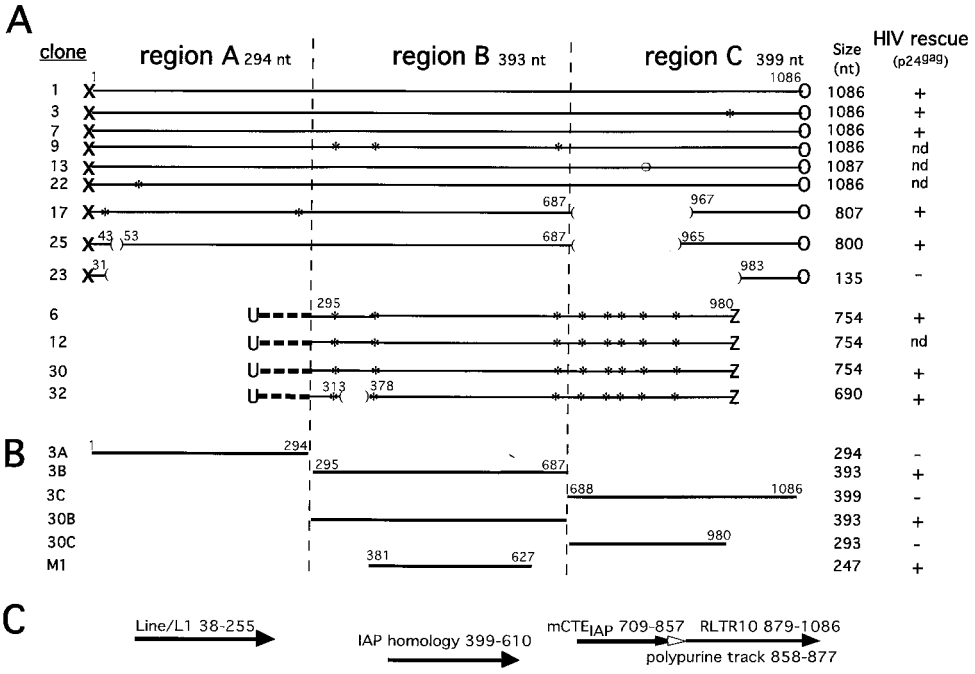


FIG. 3. Cloning and analysis of mouse DNA inserts in NL43Rev$^-$R$^-$. (A) DNA was isolated at day 14 from the cultures shown in Fig. 2A and analyzed for the presence of inserts within the HIV *nef* open reading frame. The amplified fragments (from Fig. 2B, lane 3) were purified from the gel as a mixture of 300- to 1,300-bp sequences and cloned to analyze the identity of the sequences able to rescue HIV-1 production. Two different groups of nearly identical sequences were obtained from a total of 13 sequenced clones. The clone numbers and the sizes in nucleotides are shown on the left and right, respectively. Analysis of the fragment boundaries with the vector (X and O versus U and Z) indicated different ligation events. The fragments are aligned to show the regions of identity among them. Asterisks, single point mutations. A single nucleotide insertion (open circle) was found in clone 13. The location of the deletions are shown (the numbering follows that for the nucleotides of the insert of clone 1). Selected fragments were tested for their ability to rescue virus after ligation to NL43Rev$^-$R$^-$, transfection into 293 cells, and then cocultivation with Jurkat cells. Virus propagation was monitored by measuring p24^{gag} production (right). nd, not determined. (B) To identify the minimal region able to replace the HIV Rev/RRE regulatory system, fragments A, B, and C from clone 3 and B and C from clone 30 and fragment M1 derived from clone 3 were amplified by PCR and ligated into pNL43Rev$^-$R$^-$. These molecular clones were transfected into 293 cells, which were cocultivated with Jurkat cells. Virus production was monitored by measuring p24^{gag} production (see also Fig. 4A), which is summarized on the right. (C) Regions of sequence homology between the rescued fragments found in the mouse genome. Homologies with Line/L1 repetitive elements (nt 38 to 255), IAP (nt 399 to 610), mCTE$_{IAP}$ (nt 709 to 857), the polypurine track (nt 858 to 877), and RLTR10 (nt 879 to 1086) were found.

quired a deletion spanning nt 337 to 357 (passage 11), which was further increased to nt 312 to 362 (passages 18 and 42). Sequence analysis showed that 8 of 16 clones at passage 18 and 11 of 13 clones at passage 42 contained the longer deletion, while none contained the wild-type element. No other mutations were found within the element. Third, we found that a smaller fragment of 247 nt (M1; nt 381 to 627) was also able to rescue virus propagation (Fig. 3B and 4B) to the same extent as fragments 3B and 30B. Therefore, the minimal RTE able to rescue Rev- and RRE-defective HIV-1 was located within 247 nt of fragment B and promoted HIV-1 expression to a similar extent as the SRV-1 CTE.

Identification of distinct genomic locations containing elements similar to the rescued RTE-containing fragment. Using the 1,086-nt sequence of clone 1 as a search string, we found that the region spanning nt 256 to 1086 has 97 to 99% identity with six distinct mouse genomic sequences in GenBank (accession no. AC002406, AJ278435, AF259072, AC005743, AC078931, and AL021127). Interestingly, in all cases region B containing the RTE is flanked at the 3′ end by a mutant CTE$_{IAP}$, a polypurine track, and RLTR10, as depicted in Fig. 3C. In addition, the RTE-containing region is always preceded by 1.2 to 1.9 kb of sequences having homologies with type D or IAP *gag* or *pol* sequences and a 5′ LTR (RLTR10). The discovery of several sequences in the genome showing strong homology to the rescued fragments excludes the possibility that the cloned elements (Fig. 3A) resulted from a complex rearrangement during virus rescue. Our data support the existence of a class of IAPs that carry the novel RTE. Such IAPs exist in several copies in the mouse genome, and virus rescue resulted in identification of two sequences containing functional RTEs.

Sequence comparisons. Fragment M1 (nt 381 to 627) was compared to GenBank sequences. This comparison revealed a conserved core element of 204 nt (nt 406 to 609) having strong similarity with the genome of some defective endogenous retroviruses belonging to the murine IAP family of retrotransposons and to a Syrian hamster retroelement (HAMIAP18C; GenBank accession no. M10134) (Fig. 5A). Alignments were generated with the Pileup program of the GCG software package (9), using the sequences identified with the BLAST homology search program. These rodent retroelements contain in many cases (M10134, M12515, AC004407, AE000664, X01172, AF027865, X97915, M18252, S74315, M18251,

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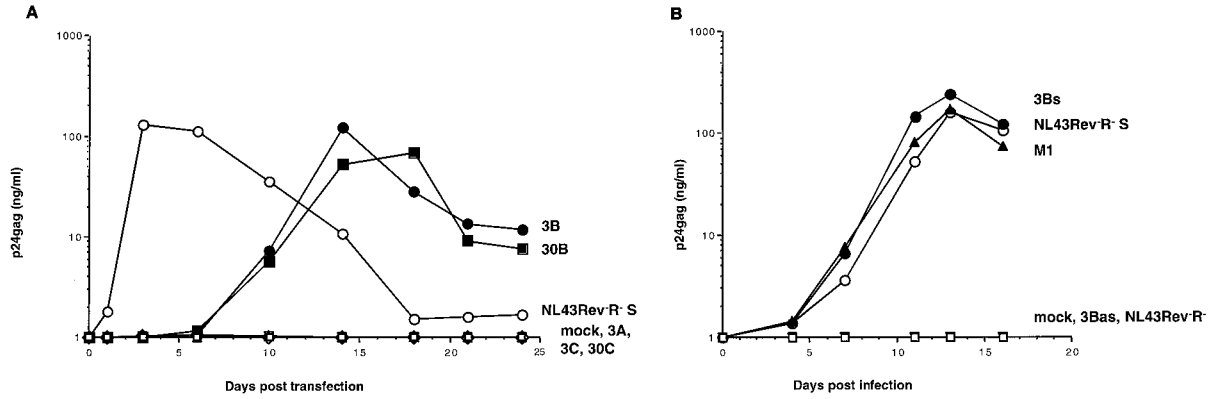


FIG. 4. Propagation of NL43Rev$^-$R$^-$ viruses with smaller fragments from region B. (A) Different fragments (A, B, C) as outlined in Fig. 3B from clones 3 and 30 were tested for function. Fragments 3A (open triangles), 3B (solid circles), and 3C (open inverse triangles) from clone 3 and fragments 30B (solid squares) and 30C (open diamonds) from clone 30 were amplified by PCR and ligated into pNL43Rev$^-$R$^-$. Upon transfection of 293 cells and cocultivation with Jurkat cells, these fragments were directly analyzed for the ability to rescue virus production. Only the presence of fragments 3B and 30B, not that of fragments 3A, 3C, and 30C, resulted in virus production (summarized in Fig. 3B). The molecular clone pNL43Rev$^-$R$^-$S (open circles) and mock transfection (open squares) served as positive and negative controls, respectively. (B) Virus can be rescued only by the presence of the RTE in sense orientation. Molecular clones of pNL43Rev$^-$R$^-$ containing fragment 3B (nt 295 to 687) inserted in sense (3Bs; solid circles) or antisense (3Bas; open triangles) orientation or shorter 247-nt fragment M1 (nt 381 to 627; solid triangles) were generated. Virus stocks generated from transfection into 293 cells and cocultivation with Jurkat cells were used for cell-free infection of fresh Jurkat cells, and virus production over time was monitored. Virus was rescued only from clones containing the RTE in the sense orientation. PCR amplification of DNA isolated from Jurkat cells infected by the 3Bs-containing recombinant HIV harvested 5 days after infection confirmed the presence of a virus containing an insert of the expected size (393 nt).

M10062, U70139, X54077, X04120, M17551, and U58494) a 3′ LTR in a fixed position downstream of the element (49 to 51 nt from the 3′ end of the core homology).

The evolutionary analysis of the aligned sequences was performed with the SplitsTree analysis tool (32) (Fig. 5B). The length of the shortest direct line connecting the sequences represents the evolutionary distance between sequences. Splits, i.e., regions of possible alternate evolutionary divergence, are indicated as rectangles (or parallel lines). The advantage of the SplitsTree program is that, unlike the GCG program Growtree, it does not invariably force the formation of a tree-like structure but is able to generate bush-like or tree-like relationships and displays possible splits based on the data.

Most of the compared sequences are clustered together very closely, while several sequences including that of RTE are evolutionarily more distantly related. In conclusion, the data bank analysis revealed the very strong similarity of the newly identified RTE to the genomes of some defective endogenous retroviruses that belong to the rodent IAP family. These IAPs have a conserved region of unknown function adjacent to the 3′ LTR. Our data show that this region contains a novel post-transcriptional regulatory element, RTE. This finding also reveals that RTE-containing IAPs can be subdivided into two classes, one which contains RTE alone and one in which RTE is adjacent to a defective CTE_{IAP} (61). Most IAPs have undergone severe mutagenesis and represent molecular fossils rather than active elements. For this reason, it is highly likely that many of the RTE-related elements identified by computer searches are inactive, as previously demonstrated for the CTE-related elements (61).

RTE promotes RNA export from *Xenopus laevis* oocyte nuclei. The ability of RTE to rescue Rev-defective HIV strains demonstrated that RTE is a potent RNA transport element capable of replacing Rev-RRE regulation by promoting the transport of unspliced and intermediately spliced RNA to the cytoplasm. To further examine the RNA transport by RTE, we inserted RTE into the intron of an adenovirus-derived mRNA precursor. Upon injection of the radiolabeled precursor RNA into the nucleus of *Xenopus* oocytes, the excised intron lariat remains in the nucleus and is exported to the cytoplasm only in the presence of an active *cis*-acting RNA transport element (14, 53, 56). In vitro-transcribed ^{32}P-labeled adenovirus precursor mRNAs containing no element, $RTE_{381–627}$ (corresponding to M1), $RTE_{391–616}$, or the type D retrovirus CTE (56) were injected together with the control U6Δss and U1ΔSm RNAs. RNA samples from total oocytes or cytoplasmic and nuclear fractions were collected either immediately or after a 3-h incubation as indicated (Fig. 6). The products of the splicing reactions were resolved on denaturing gels. Figure 6 shows that the intron lariats remained almost exclusively in the nucleus in the absence of any additional sequence (lanes 1 to 3), as expected (22). In contrast, the presence of the RTE led to significant export of the excised intron lariat, which was detectable after 3 h of incubation (lanes 7 to 9 versus lanes 4 to 6). Both $RTE_{381–627}$ (lanes 7 to 9) and shorter element $RTE_{391–616}$ (lanes 13 to 15) promote the export of the excised intron lariat with efficiencies comparable to that of the simian type retrovirus CTE (lanes 10 to 12). The coinjected U1ΔSm was found in the cytoplasm only after incubation of the oocytes (lanes 4 to 6 versus lanes 7 to 9, as well as lanes 1 to 3 and 10 to 15), while the U6Δss RNA remained in the nucleus, as expected (23, 34, 67). Taken together, these data demonstrate that RTE is a bona fide RNA transport element, which functions in *Xenopus* oocytes in addition to mammalian cells.

RTE-mediated export is CRM1 independent. To date, two pathways for nucleocytoplasmic mRNA transport have been characterized. The first utilizes nuclear export receptor CRM1,

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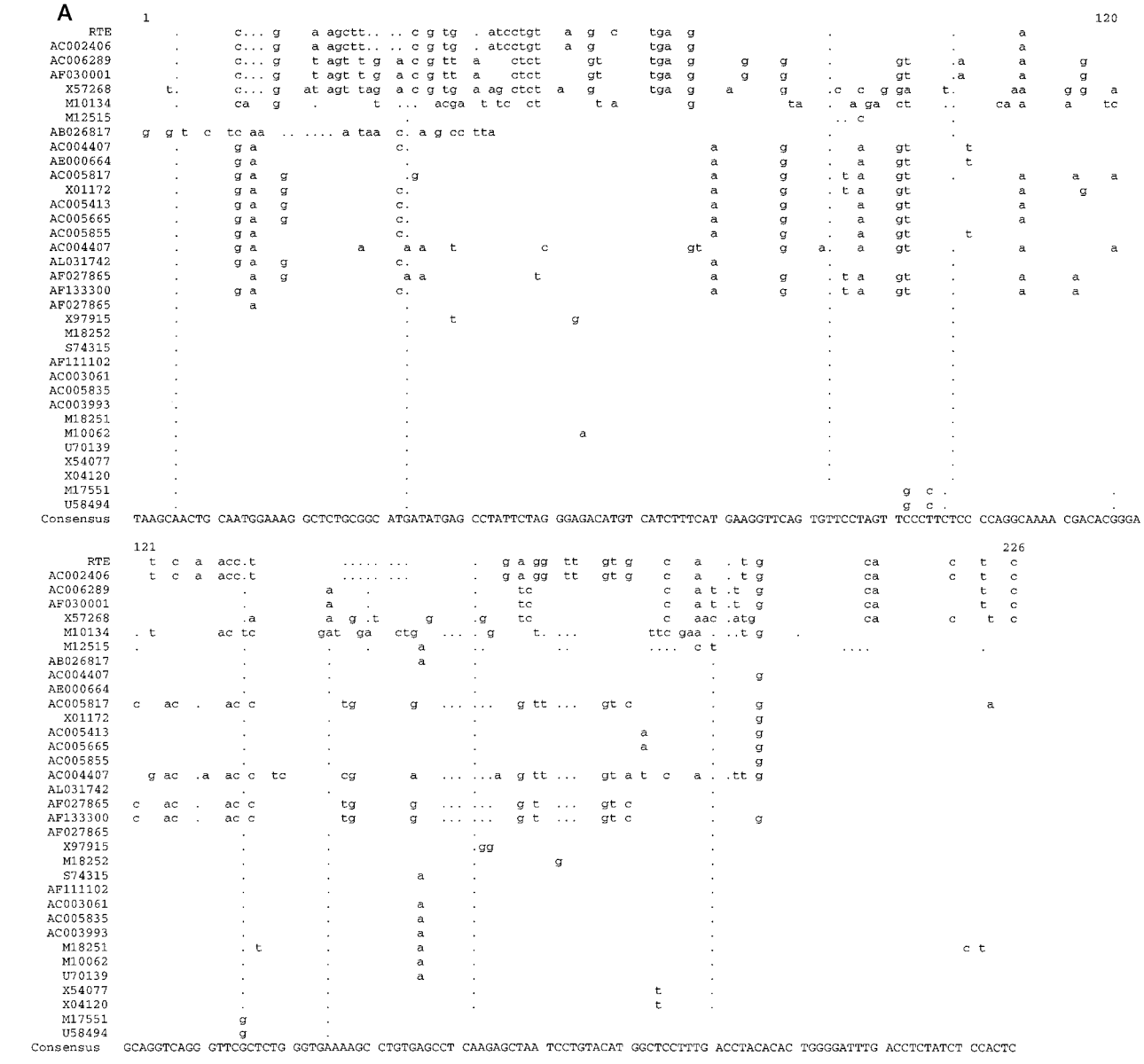


FIG. 5. Identification of RTE-related elements in mice and Syrian hamsters. (A) Sequence alignment of the core region of fragment B (nt 406 to 609) with homology to a class of IAP sequences. The functional RTE is shown at the top. Analysis was with the University of Wisconsin Genetics Computer Group software package. All sequences are from mice except M10134, which is from a Syrian hamster. (B) Evolutionary relationship of the IAP-related sequences predicted to act as RTEs. Analysis was performed with the SplitsTree software.

which binds Rev and promotes export of lentivirus mRNAs, whereas the second pathway utilizes the conserved TAP/NXF1 RNA binding protein and is used by many (if not the majority of) cellular mRNAs. To further investigate the mechanism of RTE-mediated export, we examined whether RTE utilizes the CRM1 export pathway, which can be inhibited by the presence of LMB (15, 16, 39, 69, 70). RTE was inserted into the intron of the CAT-encoding mRNA produced from indicator vector DM138. CAT expression after transfections of the resulting

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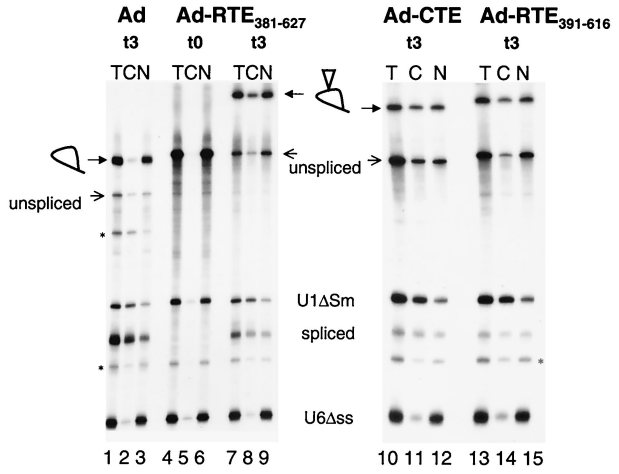


FIG. 6. RTE promotes RNA export from the nucleus of *X. laevis* oocytes. Adenovirus-derived precursor RNAs containing the RTE (Ad-$RTE_{381–627}$ and Ad-$RTE_{391–616}$) or the 173-nt CTE of SRV-1 (Ad-CTE [56]) were generated. Upon injection of *Xenopus* oocyte nuclei with the radiolabeled precursor RNA, total (T), cytoplasmic (C), and nuclear (N) fractions were prepared either immediately (lanes 4 to 6) or after a 3-h incubation (lanes 1 to 3 and 7 to 15). The injected RNA mixture also contained U1ΔSm RNA as an indicator of RNA export and U6Δss, which remains in the nucleus serving as an indicator of nuclear integrity (34). The positions of the precursor RNAs and the products of the splicing reactions are indicated on the left and in the middle. Loops, lariats; open triangles, presence of the RNA transport elements; asterisks, positions of RNA molecules thought to originate from degradation of the intron lariat and of the precursor RNA (56).

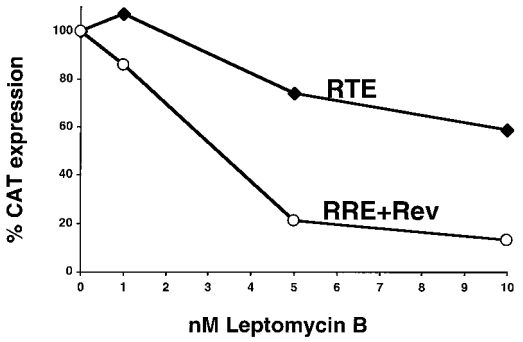


FIG. 7. RTE does not utilize the CRM1 pathway. Monkey COS-7 cells were transfected with pDM138-RTE or the RRE-containing pDM128 plus pCMVRevsg25 (59). CAT expression was measured after incubating the cells with increasing concentrations of LMB. The mean values of duplicate experiments are shown.

plasmid into cells directly reflects export of the unspliced RNA (26, 27). CAT expression vectors containing either the RRE (pDM128) in the presence of Rev or the RTE (DM138-RTE) were transfected into monkey COS-7 cells, resulting in similar levels of CAT activity (10- to 20-fold activation). Cultivation of the transfected cells in the presence of LMB (Fig. 7) resulted in potent inhibition of the Rev-mediated expression of the RRE-containing DM128, as expected (4, 51, 70), while it only marginally affected RTE-mediated CAT expression. At 5 nM LMB, 80% of the Rev-mediated expression but only 20% of RTE-mediated expression was inhibited. This significant difference in LMB-mediated inhibition is also maintained at even higher concentrations (10 nM). Weak inhibition by high concentrations of LMB was also found for the CTE-mediated expression (data not shown) (51). The observed weak inhibition by LMB of RTE- or CTE-mediated expression is thought to reflect the general toxicity of LMB (70), which blocks the function of CRM1, a key nuclear export receptor. Similar data were obtained by measuring Gag expression using RTE-containing molecular clone pNL43$Rev^{-}R^{-}M1$ instead of the CAT-expressing vector (data not shown). These results show that RTE, like the CTE, is not exported through the CRM1 pathway.

RTE function is conserved. We further tested RTE-mediated expression in several cell types using the CAT indicator vector. We found that RTE efficiently promoted CAT expression in human 293 (Fig. 8A), HeLa, and monkey COS-7 cells (Fig. 7), as well as in quail QCL-3 cells (Fig. 8B). Taken together with our infection studies using Jurkat cells and primary lymphocytes (Fig. 2 to 4) and with the RNA export assay with *Xenopus* oocytes (Fig. 6), we conclude that the RTE functions in various cell types of different species. Hence, the mechanism of RTE function is well conserved.

A comparison of RTE versus CTE function showed that RTE and CTE promote CAT expression to similar extents (10- to 20-fold activation) in human 293 cells (Fig. 8A) as well as in monkey COS-7 cells (Fig. 7). In addition, RTE and CTE function to similar extents in virus rescue experiments with PBMCs and Jurkat cells (Fig. 4) and in the RNA export assay with *Xenopus* oocytes (Fig. 6). However, we found that RTE also promoted CAT expression efficiently in the quail QCL-3 cells (Fig. 8B), which are refractory to CTE function (35). Importantly, this finding suggested that either the quail TAP/NXF1 homolog has stronger affinity to RTE than to CTE or, alternatively, that another factor, and not TAP/NXF1, might act as the primary RTE-binding factor.

RTE does not bind to TAP/NXF1 with high affinity. To further explore the role of TAP/NXF1 as an RTE-binding factor, we probed its direct binding to RTE in vitro. It has been shown that TAP/NXF1 binds RNA nonspecifically, whereas it binds to CTE in a structure-specific manner (5, 21). We therefore compared the affinities of TAP/NXF1 to CTE RNA and to RTE RNA using competition binding and mobility shift assays. When CTE was used as a radioactive probe (Fig. 9A), unlabeled CTE competed efficiently for binding to TAP/NXF1, whereas RTE did not compete for binding. CTE_{M36}, which lacks the TAP/NXF1 binding sites and which was previously shown to be inactive and to have reduced affinity for TAP/NXF1 (21), competed poorly, as expected. As additional controls, we included histone H4 mRNA (H4), which had been shown to bind to TAP/NXF1 with low affinity (5), and a mixture of random RNAs produced by in vitro transcription of total human pancreatic cDNA (data not shown). No competition was detectable using either RTE or H4 (Fig. 9A) or random RNA (not shown). In summary, the following order of competition potency was observed: CTE $\gg$ CTE_{M36} $>$ RTE = histone H4 = random RNA. There were no differences in affinity for TAP/NXF1 between RTE and random RNA identified by this assay.

To examine whether the lack of competition between CTE and RTE was due to RTE's binding to a different site on

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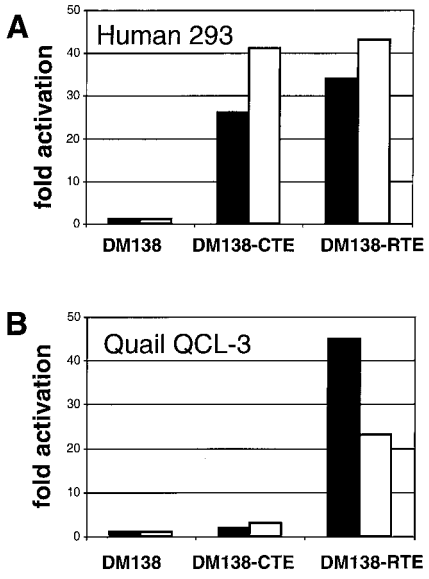


FIG. 8. RTE function is conserved. Quail QCL-3 and human 293 cells were transfected with 1 μg of pDM138, which lacks a posttranscriptional control element, and pDM138-CTE and pDM138-RTE, which contain the CTE and RTE, respectively. Two days later the cells were harvested and analyzed for CAT production. The CAT activity produced by pDM138 is normalized to 1. The mean values of duplicate transfections are given (the individual values differed by less than 10%), and two independent experiments are shown (white and black bars).

TAP/NXF1, we further performed competition assays using RTE as a radioactive probe (Fig. 9B). We found that RTE can form an apparently 1:1 complex with TAP/NXF1 with a dissociation constant of $\sim 10^{-7}$ M, which is approximately 1 log unit lower than the reported binding constant obtained using the CTE (21; our unpublished results). The unlabeled RTE and a nonrelated RNA obtained by in vitro transcription of a Bluescript plasmid (BS) similar to the one described in reference 58 competed for TAP binding with similar efficiencies, whereas the CTE RNA competed more strongly: CTE > RTE = BS. Therefore, we conclude that CTE and RTE use the same binding region on TAP/NXF1 but that RTE exhibits lower affinity, which is similar to that of a nonspecific control RNA. Taken together, these data show that TAP/NXF1 binds to RTE with low affinity and in a nonspecific manner; therefore it is not likely the primary RTE binding factor.

DISCUSSION

In this report, we demonstrate the establishment of a selection procedure for the identification and isolation of novel potent posttranscriptional control elements. We used a Rev- and RRE-deficient molecular clone to identify *cis*-acting cellular sequences that can replace the Rev/RRE regulatory system by promoting posttranscriptional activation of HIV-1, resulting in efficient virus production. The defect in this molecular clone could be compensated by the presence of heterologous transport elements such as the CTE of the type D retroviruses, the CTE-related element from an IAP (61, 62, 74), and, as shown in this work, by novel element RTE present in the mouse genome. Therefore, the use of a Rev- and RRE-deficient HIV trap represents an excellent tool for the discovery of potent RNA transport elements.

Analysis of the vector insert boundaries of the RTE-containing fragments showed that products of at least two different ligation events were propagated during the virus rescue experiments, but only a single type of element was identified. Since other functional posttranscriptional regulatory elements such as CTE_{IAP} are known to be present in the mouse genome (61), the results of this initial search are not exhaustive, and additional rounds of ligation and propagation may identify additional elements. The isolation of a single type of element multiple times may suggest that there are few elements in the genome that are sufficiently strong to be isolated by this molecular trap. In fact, the association of the identified element with IAP retroelements suggests that this type of element is functional in a retrovirus/retrotransposon setting. It is possible that only very strong elements linked with retroviruses may be uncovered with this procedure, which may be too stringent for the identification of weaker elements not allowing for virus rescue or producing very low levels of virus. Alternatively, the specific conditions we have applied may have favored one type of element. The generation of many independent pools of propagating virus and different culture conditions may allow the isolation of additional elements.

Sequence analysis demonstrated that the identified functional element has strong homology with some IAP sequences. A-type particles encoded by the endogenous proviruses are assembled on the membrane of the endoplasmic reticulum and bud into the cisternae, hence their name, IAP (for a review see reference 42). There are several hundreds of IAP copies in the rodent genome. Several of them were shown to carry the CTE-related (CTE_{IAP}) posttranscriptional control sequence (61).

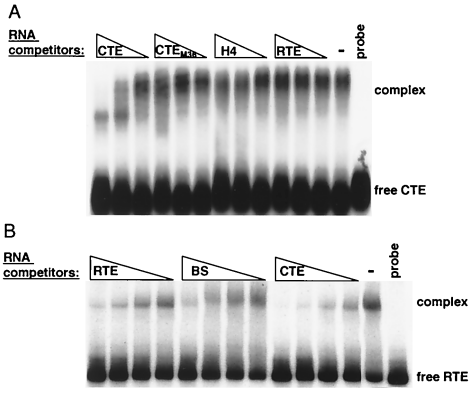


FIG. 9. Binding competition experiments. Mobility shift assays in the absence (−) or in the presence of the indicated unlabeled competitor RNAs. The assays were performed in the presence of 2 μg of heparin/ml. The positions of complexes and the free probe are indicated to the right. Probe, control without protein. (A) GST-TAP61–610 was bound to a radiolabeled CTE probe in the absence of competitor or in the presence of the indicated competitor RNAs (CTE, CTE_{M36}, histone H4, RTE) at 300, 75, or 19 nM. The reaction products were separated on a 1% agarose gel. (B) GST-TAP61–372 was bound to a radiolabeled RTE probe in the absence of competitor or in the presence of the indicated competitor RNAs (RTE, Bluescript [BS], CTE) at 267, 133, 67, and 33 nM . The reaction products were separated on a 4% polyacrylamide gel.

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Interestingly, the identification of RTE led to the definition of a new subgroup of IAPs that carry the RTE either alone or together with a nonfunctional CTE ($mCTE_{IAP}$). Furthermore, the discovery that RTE is located in a distinct group of IAP retroelements reinforces the conclusion that posttranscriptional regulation is an essential function for the expression of IAPs and many retroviruses. The RTE is located at the 3′ end of the IAP DNA and, if present alone, at a fixed distance from the 3′ LTR, which is analogous to the CTE location in type D retroviruses and IAPs.

IAP RNAs are abundant in many primary and transplanted mouse tumors and tumor-derived cell lines; they also appear regularly in early mouse embryos (72) and in certain tissues, notably the thymus (41). IAP proviral elements can act as endogenous mutagens by transposition to new locations in the genome, both at the somatic cell (8, 20, 24, 40, 43) and germ line levels (7). Transposition of IAP elements is occasionally associated with cellular transformation because they can activate the genes located near the transposition site (2, 3, 8, 11, 38, 71). Modification of cellular expression by insertional mutagenesis may be the result of gene disruption and loss of function due to insertion. In contrast, increased expression of a gene can be the result of promoter insertion. In addition, increased gene expression can be the result of mRNA stabilization due to a functional posttranscriptional element, since it is now known that IAPs contain posttranscriptional control elements such as CTE (61) and RTE (this work). The distribution of the factors binding to RTE or CTE in tissue may determine the cell types affected by such insertions. For RTE, our data indicate that the RTE-binding factor is widely distributed. The RTE has no sequence homology to any other characterized viral posttranscriptional RNA elements. Like these elements, RTE appears to have a strong and conserved secondary structure, as indicated by structure analysis (unpublished data). Comparisons of the experimentally determined structures failed to identify any structural elements in common with other functional RNA transport elements, indicating the possibility that the cellular factor binding RTE is different from those associated with the other elements.

The mechanism of RTE function is not fully elucidated. Further functional analysis may uncover additional cellular factors participating in mRNA export. We therefore asked whether RTE could be shown to work via either of the two known mRNA export pathways defined by the CRM1 and TAP/NXF1 factors. Examination of the mechanism of function of RTE revealed that it does not use the CRM1 transport pathway, since RTE export is not inhibited specifically by LMB, a CRM1 inhibitor. TAP/NXF1 not only is the export factor for the CTE-containing mRNAs but also is recognized to act as a general mRNA export factor. Our in vitro binding experiments showed that RTE and random RNA have similar affinities for TAP/NXF1. Therefore, there is no evidence of specific binding of TAP/NXF1 to RTE, indicating that the primary RTE-binding factor in the nucleus may be different. In addition, a study of RTE function in quail cells showed that RTE is fully functional in these cells, while CTE is not. This result is also consistent with the hypothesis that the primary RTE-binding factor is distinct from TAP/NXF1. The role of TAP/NXF1 in RTE-mediated export, if any, is presently unclear. RTE may define a new RNA export mechanism, which involves a distinct binding factor(s). Further characterization of the RTE will provide the tools to dissect its export mechanism. Identification of the cellular factor(s) participating in RTE function will provide a valuable tool to further explore their mechanisms of function in cellular RNA export, as has been the case for the CTE-TAP/NXF1 pathway.

In conclusion, our results provide a method for the rapid isolation of functional posttranscriptional regulatory elements from the mammalian genome. The identification and characterization of such elements will further aid the understanding of mechanisms involved in mRNA export.

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