Mapping of the Functional Boundaries and Secondary Structure of the Mouse Mammary Tumor Virus Rem-responsive Element*

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Mouse mammary tumor virus (MMTV) is a complex retrovirus that encodes at least three regulatory and accessory proteins, including Rem. Rem is required for nuclear export of unspliced viral RNA and efficient expression of viral proteins. Our previous data indicated that sequences at the envelope-3′ terminal repeat junction are required for proper export of viral RNA. To further map the Rem-responsive element (RmRE), reporter vectors containing various portions of the viral envelope gene and the 3′ long terminal repeat were tested in the presence and absence of Rem in transient transfection assays. A 476-bp fragment that spans the envelope-long terminal repeat junction had activity equivalent to the entire 3′ end of the mouse mammary tumor virus genome, but further deletions at the 5′- or 3′-ends reduced Rem responsiveness. RNAse structure mapping of the full-length RmRE and a 3′-truncation suggested multiple domains with local base pairing and intervening single-stranded segments. A secondary structure model constrained by these data is reminiscent of the RNA response elements of other complex retroviruses, with numerous local stem-loops and long-range base pairs near the 5′- and 3′-boundaries, and differs substantially from an earlier model generated without experimental constraints. Covariation analysis provides limited support for basic features of our model. Reporter assays in human and mouse cell lines revealed similar boundaries, suggesting that the RmRE does not require cell type-specific proteins to form a functional structure.
indicate that the secondary structure is a critical factor for proper function of retroviral response elements (18), and that multiple stem-loops are required. Export proteins multimerize on these elements to allow activity (19).

In the current study, we have used deletion mutations within a reporter vector based on the 3’-end of the MMTV genome to define a 476-nt element necessary for maximum Rem responsiveness. This element spans the envelope-LTR junction of the MMTV genome as previously reported (1). However, a secondary structure model generated using digestions of the RmRE by RNases V1, T1, and A as experimental controls suggests significantly from the published structure (11) and more closely resembles complex retroviral response elements. Transfection experiments indicated that the MMTV RmRE could function in both mouse and human cells, suggesting that conserved cellular proteins interact with Rem.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—XC rat fibroblast cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, gentamicin sulfate (50 μg/ml), penicillin (100 units/ml), and streptomycin (50 μg/ml). Jurkat human T lymphoma cells were maintained in RPMI media supplemented with 5% fetal calf serum, gentamicin sulfate (50 μg/ml), penicillin (100 units/ml), and streptomycin (50 μg/ml).

XC cells were transfected in 6-well plates using DMRIE-C transfection reagent (Invitrogen) according to the manufacturer’s instructions. Each transfection contained 250 ng of the reporter vector, 250 ng of pGL3-control plasmid, and 2 μg of an expression vector for Rem or green fluorescent protein (GFP). Jurkat cells were transfected by electroporation using a BTX ECM600 instrument. Cells (1 × 10^7) were mixed with the appropriate plasmid DNA in a volume of 400 μl of Jurkat medium prior to electroporation in 4-mm gap cuvettes (260 V, 70 °C prior to luciferase assays.

**Plasmid Constructs**—The plasmids GFP-Rem (RemP71), HMRluc, and HMΔeLTRluc have been described elsewhere (1). The plasmid pEGFPN3 was obtained from Clontech and the pGL3-control plasmid was obtained from Promega. The mutant HMΔeLTR+Xluc constructs were made by insertion of the mutant RmRE (X) into an engineered Scal site downstream of the splice acceptor site and upstream of the simian virus 40 poly(A) signal in HMΔeLTRluc (Fig. 1). In vitro transcription vectors for wild-type and mutant RmREs were made by PCR amplification of the wild-type or mutant RmRE with insertion of a T7 polymerase promoter upstream of the RmRE. The PCR product was then inserted into the multiple cloning site of the pEGFPN3 vector. The 1–348 mutant containing the 3’-truncated RmRE was amplified with the following primers: T7HM+ and 5’-TAA TAC GAC TCA CTA TAG GGA TCT TAA CGT GCT TC-3’ and RmRE 348, 5’-AGT ACT GTG GTC CTT GCC TCA GGA GG-3’. Cloning of the GFP-RemP71L expression vector and all other mutant RmRE constructs was performed using site-directed mutagenesis or by cleavage with restriction enzymes and religation. Details are available upon request. All constructs were confirmed by automated sequencing reactions.

**Luciferase Assays**—Luciferase assays were performed using the Dual Luciferase Reporter Assay system (Promega) to quantitate both Renilla and firefly luciferase activities (20). A firefly luciferase reporter vector lacking MMTV sequences was added to each transfection, and activities showed that different transfections within the same experiment had similar DNA uptake. Samples of normalized and unnormalized data have been provided (see supplemental Table S1 compared with Fig. 2).

**Western Blotting**—Protein extracts for Western blotting were obtained as previously described (1). Western blots were performed using antibodies specific for the GFP tag (Clontech) on GFP-Rem or actin (Calbiochem); the latter served as a control for protein loading. Proteins were detected using the ECL Western blotting detection system (Amersham Biosciences).

In *Vitro* Transcription and End Labeling of RNAs—DNAs encoding the wild-type and truncated RmREs (1–496 and 1–348) were linearized using Scal (New England Biolabs). RNAs were prepared by *in vitro* transcription using T7 RNA polymerase and purified using a Qiagen RNeasy column following the manufacturer’s protocol. RNAs were incubated with shrimp alkaline phosphatase (Promega) to remove the 5’-triphosphate and then with [γ-32P]ATP (PerkinElmer Life Sciences) and T4 polynucleotide kinase (New England Biolabs) to add a radiolabeled phosphoryl group to the 5’-end of the RNA (21). RNAs were 3’-end labeled using [5’-32P]pCp and T4 RNA ligase to extend the 3’-end of the RNA with a single-labeled nucleotide (22). Both 3’- and 5’-labeled RNAs were purified using 8% native PAGE.

**Nuclease Mapping of RmRE RNAs**—RNase footprinting reactions (10 μl) were performed using *in vitro* transcribed full-length (1–496) or 3’-truncated (1–348) RmRE RNA. Labeled RNA (3–15 nm, 10,000 cpm/μl) was incubated in 50 mM sodium/MOPS (pH 7.0) and 10 mM MgCl2. Varying the preincubation time did not affect the results (15 s to 5 min), suggesting that the RNA formed the secondary structure rapidly and stably. Nucleases were then added at levels empirically determined to give optimal RNA cleavage: 0.1 unit of RNase T1 (Sigma), 10–4 units of RNase V1 (Ambion), and 10–3 units of RNase A (Sigma). RNA was digested for 1 to 3 min at 25 °C, and aliquots were quenched by adding 1 mg/ml proteinase K and then 2 volumes of 20 mM EDTA in loading dye (90% (v/v) formamide, 0.04% xylene cyanol, and 0.04% bromphenol blue). Reaction tubes were immediately placed in liquid nitrogen to ensure that the RNases were fully inactivated. RNA sequencing ladders were generated by digesting 5’- or 3’-labeled RNA with 0.1 unit of RNase T1 for 15 min under denaturing conditions (7 M urea at 50 °C). Reaction products were separated by 8% denaturing PAGE and quantitated using Semi-Automated Footprinting Analysis software (23). From each digestion, raw intensity values corresponding to cleavage at each nucleotide were normalized by dividing each value by the average intensity of all bands within the range quantitated. These normalized values were
Mapping of the MMTV RmRE

![Diagram of reporter vectors used to map the MMTV RmRE.](image)

**FIGURE 1.** Diagram of reporter vectors used to map the MMTV RmRE. The cytomegalovirus promoter, the Renilla luciferase gene, the MMTV 3’ LTR, and simian virus 40 (SV40) polyadenylation sequences are shown as gray, black, white, and hatched boxes, respectively. Splice donor (SD) and splice acceptor (SA) sites also are shown within the MMTV-derived sequences. The smaller black box in pHM\(\Delta e\)LTR+XR\(\text{Rluc}\) represents the \(X\) number of MMTV sequences inserted in the reporter vectors to characterize the limits of the RmRE.

then averaged between experimental determinations to produce final values. Except where indicated, all reported results reflect the averages of two to six independent determinations.

**RESULTS**

The RmRE Maps to a Large Region Spanning the Junction of the MMTV Envelope Gene and the 3’ LTR—To determine the boundaries of the MMTV RmRE, several constructs were designed based on our previously described pHM\(\text{Rluc}\) vector (1, 24). This vector contains the 3’-end of the MMTV genome, including part of the envelope gene and the 3’ LTR, downstream of the cytomegalovirus promoter (Fig. 1). Because the Renilla luciferase gene was inserted between the splice donor and acceptor sites in the envelope gene, detection of luciferase activity in transfected cells indicates export of unspliced mRNA from the nucleus to the cytoplasm. Previous data showed that reporter gene activity from this vector is induced by co-expression of the MMTV export protein, Rem, in trans (1). Rem-induced luciferase activity also required the presence of the envelope-3’ LTR junction in the reporter vector, suggesting that these sequences contain the RmRE (1). The pHM\(\Delta e\)LTR\(\text{Rluc}\) plasmid, which substitutes the simian virus 40 polyadenylation region for the MMTV 3’ LTR (Fig. 1), shows no response to the addition of Rem expression vectors (1). Therefore, various MMTV sequences at the envelope-LTR border were re-inserted into the pHM\(\Delta e\)LTR\(\text{Rluc}\) vector to determine the region necessary for Rem responsiveness.

Our previous experiments showed that insertion of a BglII to Scal fragment (496 bp) within the pHM\(\Delta e\)LTR\(\text{Rluc}\) plasmid gave the same Rem response in mouse cells as the wild-type pHM\(\text{Rluc}\) vector, which contains the entire 3’-end of the MMTV genome (1). In agreement with these results, both reporter plasmids showed about 5-fold increases in luciferase activity after co-expression of Rem in transient transfections of XC rat cells (compare pHM\(\text{Rluc}\) to the 1–496 vector in Fig. 2A). Unlike mouse cells, XC rat cells lack endogenous Mtv proviruses, which may express virally encoded proteins (7). Western blotting was also used to verify expression of the Rem construct (data not shown).

Subsequent plasmids were designed to determine the 5’ border of the response element (RmRE). Deletion of 10 or 20 nt from the RmRE 5’-end had no reproducible effect on Rem responsiveness (constructs 11–496 and 21–496) (see Fig. 2A and Table 1). However, reporter plasmids with a deletion of 30 bp from the 5’-end of the vector showed a 2.5-fold decrease in reporter levels after Rem expression, and constructs with a deletion of 40 bp or more had little or no detectable Rem response (see Fig. 2A and Table 1).

Deletions also were performed to remove sequences from the 3’-end of the RmRE. Removal of 98 or 123 bp at the 3’-end of the RmRE of the reporter vector resulted in a 2-fold drop in Rem responsiveness, and deletion of 148 bp essentially showed no Rem response (Fig. 2B). Furthermore, a reporter construct carrying an internal deletion of the RmRE between 50 and 369 bp (Δ50–369) had little detectable Rem response (Table 1). These results suggest that full RmRE responsiveness requires the majority of the BglIII-Scal fragment spanning the envelope-3’ LTR region.
The MMTV RmRE Has Extensive and Complex Secondary Structure—After defining the functional boundaries of the RmRE, we used RNase mapping to probe its secondary structure. RNA spanning the wild-type C3H MMTV RmRE (nt 1–496) was produced by in vitro transcription, 5′- or 3′-end labeled with 32P, and subjected to limited digestion with RNases T1, A, and V1. RNase T1 cleaves preferentially at single-stranded G residues, RNase A cleaves at single-stranded C or U residues, and RNase V1 primarily digests base-paired nucleotides without strong preferences for nucleotide identity (25).

Thus, RNase probes used in combination give extensive information on base pairing status throughout a structured RNA (26, 27).

Results from digestions with each RNase are shown for a portion of the RmRE (Fig. 3). The RNA includes extensive secondary structure, as indicated by localized regions that were cleaved efficiently by RNase V1 and were inaccessible to RNases A and T1 (e.g. nt 199–200 and 231–234) (Fig. 3A). On the other hand, these segments were limited to a few consecutive nucleotides and were interrupted by local regions of accessibility to the single strand-specific RNases, suggesting a structure in which short helical segments are separated by many hairpin and/or internal loops.

To evaluate the RNase mapping results quantitatively, we used the freely available software Semi-Automated Footprinting Analysis (23) to determine the intensity of RNase-mediated cleavage at each position. Digestion profiles for the three RNases are provided (Fig. 3B) across the range shown in the gel (Fig. 3A). Profiles for the entire RmRE also are shown (supplemental Figs. S1–S3). From the average profiles of multiple independent determinations, we established empirically two threshold levels of intensity. Bands that significantly exceeded the average band intensity were considered to reflect nucleotides that were accessible to each nuclease. A second, higher threshold was also established to separate the smaller groups of nucleotides that were the most accessible to each RNase probe (supplemental Figs. S1–S3).

With the latter set of the most reproducible and largest signals as experimental constraints, we used Mfold to generate possible secondary structures of the RmRE (28). The structure predicted to be most stable, after using constraints from the RNase digestions, is shown (Fig. 4). This structure is highly complex, containing many single-stranded regions and multiple hairpin loops typical of the response elements of other complex retroviruses (12, 26, 29–31). This structure has been divided into regions I, II, III, and IV. Also similar to other response elements, the secondary structure models favored by Mfold include long-range base pairings between sequences close to the 5′ and 3′ boundaries of the RmRE within region I (Fig. 4 and data not shown).

Interestingly, in the absence of experimental constraints, Mfold predicted secondary structures that differed substantially from that shown in Fig. 4. The most favorable structure in the absence of the constraints is shown for comparison (see supplemental Fig. S4). Although this structure retains the most basic features of the model in

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**TABLE 1**

| RmRE construct | Relative Rem response |
|----------------|-----------------------|
| Wild-type (1–496) | 100 ± 14^b |
| 11–496 | 110 ± 21 |
| 21–496 | 108 ± 38 |
| 31–496 | 39 ± 7.1 |
| 41–496 | 0 |
| 55–496 | 0 |
| 1–398 | 50 ± 4.0 |
| 1–373 | 51 ± 11 |
| 1–348 | 21 ± 4.7 |
| Δ50–369 | 8.9 ± 0.2 |

^a Average luciferase activities of triplicate mutant transfections in XC cells are given relative to the wild-type (WT) RmRE construct (± S.D.).

^b The Rem-dependent increase for the wild-type plasmid was 5–7-fold, which was designated as 100%.
Fig. 4, multiple stem-loops and long-range pairings between sequences near the boundaries, local interactions differ substantially. Importantly, the model generated by including experimental constraints provides better agreement with the footprinting data that were not used as constraints. Of 43 nucleotides that were reactive to RNase V1, but were not constrained in the structural prediction, 72% are correctly predicted to be double-stranded, compared with only 35% for the unconstrained model (data not shown). The agreement with RNase A data were comparable for the two models (41% of reactive nucleotides are single-stranded in the model generated with constraints versus 35% for the model without constraints), whereas, for RNase T1, only six reactive nucleotides were not used as constraints, preventing meaningful analysis.

When all of the footprinting data are considered, the constrained model gives much higher rates of agreement for all three RNases. Although this is true by necessity because many of the reactive nucleotides were constrained in the modeling, this comparison nevertheless highlights the point that a secondary structure may be obtained to give much stronger agreement with experimental results than the one that would be favored in the absence of experimental constraints.

The Limits of the MMTV RmRE Are Unaffected by Cell Type—Previous experiments suggest that Rem is synthesized as a 33-kDa precursor protein, which is targeted by an unusually long signal peptide (SP) (98 amino acids) for translocation across the endoplasmic reticulum membrane (1, 32). Subsequently, the Rem C terminus is glycosylated, and the N-terminal SP appears to be cleaved by signal peptidase in the endoplasmic reticulum lumen. Our previous data showed that GFP-tagged Rem accumulates in nucleoli (1). Experiments by Dultz et al. (32) showed that cleaved Rem SP is released into the cytoplasm prior to nuclear localization. We have
observed similar results using cells transfected with Rem cDNA tagged on the N terminus with GFP; the major product obtained was consistent with the size of GFP plus the Rem SP (~38 kDa) (Fig. 5A). Furthermore, GFP-tagged Rem constructs with a leucine instead of proline at position 71 (P71L) had greater induction of reporter activity with the 1–496 construct (~24-fold) (Fig. 5B) compared with RemP71 (5–7-fold) (Fig. 2).

GFP-tagged RemP71L then was tested with 5′-deletion mutants of the RmRE. As noted with the less active version of the protein (Fig. 2), deletion of 30 bases from the 5′-end of the RmRE was responsive to Rem. Although the basal activity of the 31–496 mutant was ~5-fold lower in some assays, the induction by RemP71L was similar to that observed for the wild-type construct (1–496). The 31–496 mutant showed about 50% of the wild-type activity using RemP71L compared with RemP71 (Table 1), which may be a consequence of proline instead of leucine at position 71. However, deletion of another 10 bases from the 5′-end of the RmRE removed all Rem responsiveness (mutant 41–496). Nucleotides 30 to 40 are essential, either because they form an important structure or because their deletion leads to larger scale rearrangements.

Mutants deleted at the 3′-end of the RmRE also were tested for their response to RemP71L. The results indicated that the 1–373 mutant had about 50% of the Rem responsiveness of the wild-type RmRE, whereas the 1–348 mutant had ~25% of wild-type RmRE activity. These experiments also agree with those obtained with RemP71 and suggest that deletions at the 3′-end of the RmRE are more easily tolerated than those at the 5′-end, perhaps because 3′-deletions are less likely to give global rearrangements.

Consistent with this interpretation, RNase footprinting of the 1–348 mutant showed substantial rearrangements of the 3′ portion of the RmRE but appeared to preserve an extensive local structural element in the 5′-half of the RmRE (nt 70–160, largely region II in Fig. 4) (also see supplemental Figs. S1–S3).

To determine whether cellular factors would affect the region required for Rem responsiveness, the same 5′- and 3′-deletion mutants were also tested in human Jurkat T cells (Fig. 5C). Like XC fibroblasts, Jurkat cells lack endogenous
MMTV proviruses that might contribute to Rem activity, yet these cells are capable of infectious virus production (33). Induction by Rem of the 1–496 construct observed in Jurkat cells was higher than that observed in XC cells, which may be due to the higher transfection efficiency of Jurkat cells. However, both the 5′ and 3′-deletions had a similar response to mapping of the MMTV RmRE.
RemP71L in each cell type (compare Fig. 5, B and C). These results suggest that the RmRE structure is intrinsic to the RNA sequence and not dependent on the presence of cell type-specific proteins.

Covariation Analysis Supports a Complex MMTV RmRE Structure with Multiple Stem-Loops—To obtain further biological evidence for the proposed RmRE structure (Fig. 4), we performed covariation analysis on nucleotide changes observed in various MMTV isolates available in public databases. Although the number of such complete sequences is relatively limited, an alignment of the RmRE regions of milk-borne MMTVs and endogenous proviruses that also can be transmitted through the milk (Mtv2 (also known as GR-MMTV) and Mtv1) was obtained (Fig. 6). Interestingly, four of the 16 sequences showed a C to U transition at nucleotide 90 that was accompanied by G to A change at nucleotide 95 (C3H/HeJ, SW, JYG, and BALB2 (bold type), its predicted base pairing partner. Furthermore, most sequences retained the ability to form a wobble base pair by including U at position 90 and G at position 95. In addition, four sequences with a G to A change at position 136 showed a C to U change at position 166 (CS, TES2, RII/Sa1, and RII/Sa2 (two isolates)) (bold and underlined type). Positions 136 and 166 are base paired in the unconstrained model (see supplemental Fig. S4). Only two sequences showed 136A and 166C (no predicted base pairing). No covariation was observed in the extended hairpin model proposed for the RmRE by Müllner et al. (11). Furthermore, a 3-nt insertion in the GR-MMTV RmRE mapped to a single-stranded portion of region IV. Thus, covariation analysis provides limited support for both the constrained and unconstrained complex stem-loop models for the MMTV RmRE (see Fig. 4 and supplemental Fig. S4), but not the extended hairpin model.

DISCUSSION

Previous experiments indicated that MMTV is a complex retrovirus that encodes the Rem regulatory protein from a doubly spliced mRNA (1, 2). Rem is required for optimal export of full-length MMTV RNA from the nucleus (1), and also for a post-export function that depends on sequences within the 3’ MMTV LTR (24) as well as the junction with the envelope gene (11, 24). In this study, we have mapped the limits of the MMTV RmRE using a reporter vector based on the 3’-end of the MMTV genome (1, 24). Although essentially all of the 496-bp RmRE region defined earlier was required for full activity, deletion of 30 bp at the 5’-end or up to 148 bp at the 3’-end gave constructs that retained partial activity in the assays.

To understand the nature of the RmRE structure, we subjected in vitro transcribed RNA to mapping with three RNase enzymes, which recognize different single-stranded or double-stranded nucleotides. The RNase protection data were then used as constraints in Mfold, and a secondary structure prediction was generated. Interestingly, the predicted structure (Fig. 4) is quite different from that previously published for the MMTV RmRE (11). The published RmRE structure, which was solely based on the Mfold algorithm (11, 28), contains predominant double-stranded regions typical of constitutive transport elements found in simple retroviruses (34–37). In contrast, our constrained structure has many single-stranded regions and a complex stem-loop structure that more closely resembles the response elements of the complex retroviruses, HIV, human T-cell leukemia virus, and human endogenous retrovirus type K (13, 16, 26, 30, 38). Recent work has shown that the HIV Rev and human T-cell leukemia virus Rex regulatory proteins can function on the MMTV reporter vector pHMRlux in human cells (24). The activity of the heterologous regulatory proteins on the vector required the presence of the RmRE. Thus, common features of the RNA structure appear to be recognized for Rem, Rev, and Rex function. Both Rev and Rex require a stem with a bulge for the initial RNA binding followed by multimerization of the protein on the response element (39, 40).

The secondary structure predicted with the inclusion of the structure mapping data is also significantly different from a prediction for the identical region in the absence of the experimental constraints. For RNAs of this size and complexity, experimental constraints are important aids in predictions of secondary structure. Even with the experimental constraints, the accuracy of the secondary structure prediction is limited. First, not all of the nucleotides are constrained by the data, and many possible structures remain compatible with the experimental results. There are also limitations in structure prediction algorithms, most notably the inability to predict pseudoknots. Furthermore, the structure of the RmRE may be influenced by its flanking sequences, by co-transcriptional folding, or by other features of the cellular environment that are not captured in vitro in structure mapping experiments. Nevertheless, the central features of the predicted structure, the extensive secondary structure, multiple stem-loops, and long-range base pairs, are supported by boundary mapping experiments and are consistent with results from other complex retroviruses (15, 16, 18, 26), suggesting that these features are likely to be present in the natural context of the RmRE.

Consistent with a structural model involving long-range base pairs between the boundary regions, extensive deletion from either the 5’- or 3’-ends to eliminate the long-range interactions severely compromised Rem responsiveness in reporter assays (see Figs. 2 and 5 and Table 1, constructs 55–496 and 1–348). On the other hand, the effects of 5’- and 3’-deletions...
Mapping of the MMTV RmRE were not equivalent. Whereas even small deletions from the 5′-end had strong effects on Rem responsiveness, more substantial deletions were tolerated from the 3′-end. For example, the 5′-deletion mutant 41–496 blocked function, but a deletion from the 3′-end, 1–373, retained significant function, even though both deletions are predicted to disrupt the same set of long-range interactions (Fig. 4). Even the more extensive deletion, mutant 1–348, retained activity in some contexts (Fig. 5). The lower sensitivity to 3′-deletions may reflect, in part, a predicted local element toward the 3′-end, which is apparently not essential for function. Alternatively, deletions from the 5′-end may lead to more extensive rearrangements in the central region, and these regions are critical for Rem responsiveness. Supporting this idea, a construct that retained the capacity to form the long-range interactions, but lacked the central region (an internal deletion of bases 50 to 369), had little Rem responsiveness in reporter assays (Table 1).

The structural consequences of the 3′-deletion mutant, 1–348, were determined. This mutant, which was minimally functional in the reporter assays, was used for RNase mapping and compared with the full-length 496-nt sequence. Quantitation and comparison of RNase VI protection assays revealed that the mutant and wild-type RmREs differed dramatically from nt 160 through the 3′-ends (see supplemental Fig. S3). Dramatic changes were also observed between nucleotides 240 and 290 using RNase T1 (supplemental Fig. S1) as well as between nt 50 and 60 and between nt 220 and 280 using RNase A (see supplemental Fig. S2). Because the reporter assays indicated that the 1–348 mutant had low levels of Rem responsiveness and that the 5′-end of the RmRE must remain intact for function, RNase protection data confirms that the majority of the 5′-end of the 1–348 sequence retains the wild-type structure. No tertiary structure has been detected for the RmRE.4 However, recent experiments have used SHAPE analysis to map the secondary structure of the HIV RRE in the presence of Rev compared with a mutant RRE selected in the presence of a Rev nuclear export sequence mutant (30). These results indicated that the RRE has considerable flexibility in structure to allow Rev binding and function, suggesting that the 3′-end of the RmRE may also have a flexible structure.

Current reports indicate that Rev first binds to a branched stem-loop (stem-loop IIA/IIB/IIC) at nt 103–106 and 123–131 of a minimal 233-nt RRE (30) and, at higher Rev concentrations, also binds to stem-loop III/IV, which may allow structural changes and binding of cellular factors. Rev multimerization on the RNA is believed to be required (41). The equine infectious anemia virus RRE and the human endogenous retrovirus type K RcRE also may bind their respective regulatory proteins in two distinct regions (15, 38).

Use of Rev peptides and an oligomer derived from HIV stem-loop IIB have indicated considerable flexibility of the RRE (42). Purified Rem protein has been difficult to produce, but preliminary data using filter-binding assays indicate that Rem SP binds with comparable affinities to both the full-length and RmRE-(1–348).4 By analogy to the RRE, secondary structural changes at the 3′-end of the mutant RmRE may hamper necessary conformational changes that allow recruitment of cell proteins. The requirement for flexibility of the response element structure is supported by experiments showing that engineering of a completely base-paired stem (10 bp) at the ends of the RmRE abolished Rem responsiveness.5

Considerable variability in size and location has been reported for the response elements that bind the Rev-like regulatory proteins of complex retroviruses. The equine infectious anemia virus RRE has been reported to be 555 nt near the 5′-end of the envelope gene (43), but a minimal 57-nt sequence that spans the exonic splicing enhancer can function in a nuclear export assay (44). The minimal HIV RRE has been reported to be 234 nt (29) and is localized to the envelope gene (29, 45), whereas the fully active element appears to be 351 nt (45). The human T-cell leukemia virus-1 Rex-responsive element is 254 nt and spans the U3 and R regions of the 3′ LTR (13, 14, 46). The full-length RcRE was reported to be 416 to 429 nt and also localized to the U3/R border (15, 16). However, like the RmRE, a 374-nt RcRE deleted from the 3′-end had partial activity (47).

Müllner et al. (11) reported that deletion of a 490-bp fragment spanning the MMTV envelope-3′ LTR junction abolished Gag expression from an MMTV molecular clone, which is consistent with our original report (1). This fragment and various deleted versions were inserted into a heterologous HIV-based reporter vector for analysis of HIV Gag expression in cat kidney cells, which lack endogenous MMTV proviruses (48). Although the response was not quantitated, a minimal element of 279 nt was shown to be Rem-responsive. Our data indicate that the RmRE-(1–348) is minimally functional, and the 3′-end of this sequence extends beyond their minimal element. Deletion of 30 bases at the 5′-end of our RmRE retained considerable activity (Figs. 2 and 5); this deletion includes stem I of their sequence, which also was not required in their mapping experiments (11). Sequences spanning their proposed stem IIA, IIB, and IIC were essential for activity in their assays as well as ours. Our data indicate that part of region I and all of regions II, III, and IV are required for full RmRE function (Fig. 4). Differences with our results and those of Müller et al. (11) could be due to the context of the sequences in the different reporter plasmids or use of different cell lines.

Our results also differ from the extended hairpin model of Müller et al. (11) using covariation analysis. A transition from C to U at nt 90 was accompanied in four MMTV isolates by a G to A change at nt 95, positions that are predicted to be base paired in the model constrained by the RNase protection data (Fig. 4). Furthermore, a G to A change at nt 136 of four additional MMTV isolates was accompanied by a C to U change at nt 166, positions that are predicted to be base paired in the unconstrained structure (supplemental Fig. S4). These data may indicate that the RmRE undergoes structural rearrangements between the unconstrained and constrained structures similar to reports with the HIV RRE (30). However, we should note that covariation analysis is limited by the availability and

4 A. Chadee, unpublished data.

5 J. Mertz, unpublished data.
Mapping of the MMTV RmRE

The boundaries of the MMTV RmRE also were tested in two different cell lines using a more highly active form of Rem, P71L (Fig. 5). Unlike the originally reported sequence (1), leucine is found in position 71 of the signal peptide in both the RIII and C3H MMTV exogenous viruses (56, 57). The presence of proline may impair Rem cleavage to SP by signal peptidase. A GFP-SP fusion, which correlates with the higher activity in reporter assays, was observed by Western blotting (Fig. 5A). Although higher reporter activity was obtained with P71L, no differences in the limits of the RmRE were obtained using either XC rat fibroblasts or human Jurkat T cells. Such results indicate that the cellular machinery that interacts with the RmRE is conserved between rodents and humans.

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