Mapping of Multiple RNA Binding Sites of Human T-cell Lymphotropic Virus Type I Rex Protein within 5'- and 3'-Rex Response Elements*

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Interaction between the human T-cell lymphotropic virus type I (HTLV-I) Rex protein and viral transcripts in the nucleus is essential to the cytoplasmic appearance of unspliced and singly spliced viral RNA. Rex has been shown to mediate its function through direct interaction with a highly ordered secondary structure in the 3'-untranslated region of all human T-cell lymphotropic virus type I mRNAs termed the Rex response element (3'-RxRE). Part of the 3'-RxRE sequence is also present in the 5'-end of viral transcripts (5'-RxRE), and we demonstrate that Rex binds to this RNA with essentially the same affinity and specificity as to the 3'-RxRE. We have analyzed the secondary structures and binding sites of Rex within the 5'- and 3'-RxREs by enzymatic probing and chemical modification interference and show that multiple Rex molecules bind within a stem-loop, which is similarly structured in the two RxREs. Our experiments confirm the presence of a previously characterized Rex binding site but also identify a common motif within an extended region that comprises an additional Rex binding site. This suggests that Rex oligomerizes on the RxREs similarly to what has been observed for binding of the human immunodeficiency virus type 1 Rev protein to the Rev response element.

Human T-cell lymphotropic virus type I (HTLV-I)† is the etiologic agent of adult T-cell leukemia and of a chronic neurologic disorder known as tropical spastic paraparesis or HTLV-I-associated myelopathy (for a review, see Ref. 1). HTLV-II is closely related to HTLV-I when comparing genome structure and nucleotide sequence, but unlike HTLV-I, no certain correlation to diseases has been established. Like human immunodeficiency virus type 1 (HIV-1), HTLV-I (and -II) is a complex retrovirus and as such its replication cycle is divided into an early nonproductive and a late virion-producing stage (for a review, see Ref. 2). During the early stage of infection, the regulatory proteins Tax and Rex are expressed from doubly spliced mRNA. Tax up-regulates transcription of viral as well as a number of cellular genes (1). Rex acts at the posttranscrip-

tional level to induce the appearance of unspliced and singly spliced viral mRNA in the cytoplasm (3–5). These RNA species encode the structural and enzymatic polyproteins Gag, Pol, and Env, and the 9-kilobase unspliced RNA serves in addition as the viral genome.

It is not yet fully clarified how HTLV-I Rex mediates the accumulation of incompletely spliced mRNAs in the cytoplasm. Recently, the leucine-rich activation domain of Rex has been shown to constitute a nuclear export signal (6–8), suggesting that Rex directly activates the transport of intron containing RNAs into the cytoplasm. The activation domain of Rex is furthermore interchangeable with the HIV-1 Rev activation domain (9, 10), which has also been characterized as a nuclear export signal (11–13). However, Rex may also directly inhibit splicing, as suggested by the observation that Rex alters the balance between unspliced and spliced RNA in the nucleus as well as in the cytoplasm of T-cells (14). In addition, it has been reported that Rex specifically prevents the unspliced RNA from degradation in the nucleus (14).

Rex function requires the presence of an RNA element, the Rex response element (RxRE), present in the 3'-untranslated region of all HTLV-I transcripts (15–18). By computer modeling (15), mutational analyses (16, 18), and RNase probing (17), this element has been shown to form a highly ordered secondary structure consisting of four stem-loops, A–D, and two stems, I and II (see Fig. 3B). Rex binds directly to the RxRE in vitro (19, 20), and mutational analyses of the RxRE have demonstrated that Rex binding in vitro correlates with Rex function in vivo (18, 21). Within the RxRE, the stem-loop D has been shown to be indispensable to Rex binding and function (16–18, 21). In accordance with this, Rex binds the stem-loop D alone in vitro (22), and modification interference studies and in vitro selection have identified a two-bulge structure within the stem-loop D as the primary binding site for Rex (22, 23).

As a consequence of the retroviral replication mechanism, part of the RxRE sequence is also present in the 5'-end of the viral transcripts. Therefore, we introduce the terms 5'-RxRE and 3'-RxRE for denoting the RxRE at the 5'- and 3'-ends of the HTLV-I RNA, respectively. Importantly, the sequence forming the stem-loop D is present at both ends of the unspliced transcript. However, since the stem-loop D of the 5'-RxRE also harbors the splice donor site used for generating the spliced mRNAs encoding Env, Tax, and Rex, these mRNAs probably do not contain a functional 5'-RxRE. Considering that Rex in part regulates the cytoplasmic appearance of unspliced mRNA species, a potential regulatory mechanism resides within the 5'-RxRE.
Insight into Rex activity also derives from studies of the related virus, HTLV-II. HTLV-II and -I Rex can functionally replace each other (15), and both viruses require the presence of a functional splice donor site in the 5′-RxRE for Rex function (25, 26). HTLV-II Rex has further been shown to inhibit in vitro splicing of an RNA substrate containing most of the HTLV-II R and U5 region upstream of a heterologous intron and exon but only if the Rex binding site within the 5′-RxRE is intact (27). Furthermore, in HTLV-II both the 5′-RxRE (26, 28, 29) and the 3′-RxRE (30) have been shown to be functionally important.

Since previous studies in HTLV-I have focused on the function of the 3′-RxRE, it remains an open question whether the 5′-RxRE plays any role in the replication cycle of HTLV-I.

In this report, we compare the RNA structures and Rex binding sites within the 5′- and 3′-RxRE in HTLV-I and conclude that both structures contain similarly positioned multiple Rex binding sites. In addition to a binding site previously defined, we identify a second Rex binding site, which comprises a homologous RNA structure motif.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The plasmids pGEM-3Zf(+)-RxRE, pGEM-3Zf(+)-RxRE encoding sequences of pGEX/GEH-Rex and pGEX/GEH-Rex M1, the GST-Rex and GST-Rex M1 plasmids encode fusion proteins consisting of the 5′-RxRE, it remains an open question whether the 5′-RxRE binds because of a substitution of three arginine residues, allowing rapid purification. All constructs were versed primer. The resultant PCR fragments were digested with 5′-RxRE encoding fragment digested with EcoRI and SalI (positions 354–563 of CR-1) was inserted into the BamHI site of pGEM-3Zf(+)-RxRE, using the oligonucleotide 5′-GCGAATTCGGCTCGCATCTCTCCTTCA-3′ with a PCR-amplified 5′-RxRE encoding fragment digested with EcoRI and SalI (positions 354–563 of CR-1). The PCR reaction was performed with the oligonucleotide 5′-GAGAATTCGCCGCTGCATCTCCTCTCA-3′ and a standard SP6 primer and pGEM-3Zf(+)-RxRE as template.

The vectors for expression of glutathione S-transferase (GST)-tagged Rex and Rex M1 were made by insertion of a PCR fragment encoding Rex or Rex M1 into the EcoRI-BamHI site of pGEX/GEH. Rex M1 is deficient in RxRE binding because of a substitution of three arginine residues in the N-terminal RNA binding domain with an aspartic acid-leucine dipeptide (Ref. 21; data not shown). The PCR was done with either a 5′-GAGAATTCGGCTGCATCTCCTCTCA-3′ oligonucleotide (for Rex synthesis) or a 5′-GAGAATTCGGCTGCATCTCCTCTCA-3′ oligonucleotide as the forward primer and a 5′-GAGAATTCGGCTGCATCTCCTCTCA-3′ oligonucleotide as the reverse primer and either pBIC2/CMV-Rex or pBIC2/CMV-Rex M1 as templates. The resulting pGEX/GEH-Rex and pGEX/GEH-Rex M1 plasmids encode fusion proteins consisting of the GST protein at the N terminus followed by a recognition site for enterokinase, the Rex or Rex M1 protein, respectively, and finally a small domain of 10 residues enabling C-terminal radiolabeling of the fusion protein with heart muscle kinase.

His6- and His12-Rex M1 vectors were made by PCR-amplifying the Rex encoding sequences of pGEX/GEH-Rex and pGEX/GEH-Rex M1, with the same forward primers as described above and a 5′-CCACCAGGCTTGGGGGCAGGGGCGAGGGGGAG3′ primer as reverse primer. The resultant PCR fragments were digested with BamHI and HindIII and ligated into the corresponding sites of the expression plasmid pDS-H6 (34). In this context, Rex (or Rex M1) is preceded by 6 histidine residues, allowing rapid purification. All constructs were verified by sequencing.

**RNA Preparation**—To generate sense RNA, pGEM-3Zf(+)-RxRE, pGEM-3Zf(+)-RxRE encoding fragment digested with EcoRI and SalI (positions 354–563 of CR-1) was inserted into the BamHI site of pGEM-3Zf(+)-RxRE, using the oligonucleotide 5′-GCGAATTCGGCTCGCATCTCTCCTTCA-3′ with a PCR-amplified 5′-RxRE encoding fragment digested with EcoRI and SalI (positions 354–563 of CR-1). The PCR reaction was performed with the oligonucleotide 5′-GAGAATTCGCCGCTGCATCTCCTCTCA-3′ and a standard SP6 primer and pGEM-3Zf(+)-RxRE as template.

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RESULTS

Rex Binds Directly to the 5'-RxRE—It is well established that purified Rex protein binds directly to the 3'-RxRE, derived from the 3'-end of viral transcripts (19–21). Part of the 3'-RxRE sequence is also present at the 5'-end of viral transcripts, and an in vitro transcript initiating from the proviral transcription initiation site (5'-RxRE, Fig. 1) was tested for Rex binding capacity. Two recombinant types of Rex protein containing either GST or His₆ tags were expressed and purified from E. coli and used for electrophoretic mobility shift assays. Initially, increasing amounts of GST-Rex was incubated with radiolabeled 3'-RxRE RNA in the presence of E. coli tRNA as nonspecific competitor and run on a nondenaturing gel (Fig. 2A, lanes 1–8). At lower Rex concentrations, two distinct complexes are visualized (Fig. 2A, lanes 2–4), the first one being formed with an estimated K₅₀ < 16 nM. At increasing concentrations of GST-Rex, gradually slower migrating complexes are retarded in the gel (Fig. 2A, lanes 5–8), indicating either the existence of multiple Rex binding sites within the 3'-RxRE or protein-protein oligomerization. To investigate the specificity of the complex formation, we made competition experiments with increasing amounts of either sense or antisense 3'-RxRE (Fig. 2A, lanes 9–14). Preincubation of GST-Rex with 125 nm unlabeled sense 3'-RxRE totally inhibited complex formation between GST-Rex and the radiolabeled probe, whereas antisense 3'-RxRE had no effect (Fig. 2A, compare lanes 13 and 14 with lane 7). Purified GST protein alone had no detectable affinity for the 3'-RxRE (Fig. 2A, lane 15). A very similar result was obtained using the 5'-RxRE as probe (Fig. 2B). Thus, GST-Rex binds with similar affinity and specificity to both RxREs.

To increase the resolution of the higher order complexes containing Rex and the 5'-RxRE, the shorter protein His₆-Rex was used in a similar mobility shift analysis. Four distinct complexes can be detected (denoted A–D in Fig. 2C), and the K₅₀ values for the first three complexes are roughly estimated to be ~10, ~50, and ~200 nM, respectively. Hence, both types of recombinant Rex bind the 5'-RxRE with similar affinity. The affinity of GST-Rex for the stem-loop D sequence alone (Fig. 1) was then examined using the same conditions as for the 5'- and 3'-RxRE, resulting in formation of two distinct complexes denoted A and B (Fig. 2D). Again the association is specific as judged from the challenge with either sense or antisense 3'-RxRE RNA (lanes 8–13), but the affinity is approximately 30-fold lower as compared with the RxREs (K₅₀ ~ 500 nM). Finally, a minimal RNA element, HB-13 (nucleotides 130–141 joined to 160–172), encompassing the nucleotides earlier shown to be critical to mediate Rex binding (22, 23) displayed >100-fold lower affinity toward GST-Rex in gel mobility shift analysis as compared with the RxREs (data not shown).

Ribonuclease Probing of Rex Binding to the 3'-RxRE—The observation of multiple complexes in gel mobility shift assays suggests the existence of more than one Rex binding site within the 5'- and 3'-RxRE as is the case in HIV-1, where multiple Rev binding sites within the Rev response element (RRE) are required for Rev function (37–39). To map the higher order binding sites within the RxREs, we performed RNA footprinting analysis. In vitro transcribed 3'-RxRE was incubated with a 50-fold molar excess of GST-Rex to generate higher order complexes (corresponding approximately to the conditions used in Fig. 2A, lane 4). As a negative control, GST-Rex M₁, which is deficient in RxRE binding (Ref. 21; data not shown) was included. The RNA was subsequently cleaved partially with ribonucleases A, T₁, T₂, and V₁, and cleavage sites in the RNA were detected by primer extension. To keep the fraction of doubly cleaved molecules very low, conditions were chosen so that more than 50% of the RNA remained uncleaved. By employing two radiolabeled primers, complementary either to the very 3'-end of the 3'-RxRE (RxRE P₁) or to positions 102–119 (RxRE P₂), the sequence of the whole RxRE could be clearly resolved. The probing was repeated three times, yielding essentially the same result as shown in Fig. 3A.

Since the RNases have different reactivities toward single- and double-stranded RNA regions, the probing gives information about the structure of the RNA. The cleavage pattern was compared with published secondary structure models (16–18), and a proposed secondary structure is depicted in Fig. 3B. RNases T₁ and T₂ cleave predominantly single-stranded RNA, and the majority of the cleavages obtained by these RNases, and in particular the most intense ones, were in loops or bulges or at the end of helices. Equivalently, with the double-stranded specific RNase V₁, we mainly saw cleavages at base-paired nucleotides, although some unpaired positions adjacent to helices were also hit, which may reflect additional structure or stacking (40). Due to the tendency of RNase A to cut double-helical regions in addition to single-stranded RNA, especially at the salt concentration used in these experiments (170 mM KCl, 30–50 mM NaCl), the observed RNase A cleavages are not included in Fig. 3B.

Several positions became specifically protected against cleavage upon GST-Rex binding (Fig. 3A; data summarized in Fig. 3C). Rex strongly affected cleavages in the A₁₂₁–G₁₃₆ and U₁₆₇–G₁₆₉ regions within the stem-loop D. Besides residues located in regions previously found to be important to Rex binding capacity (the regions G₁₃₀–G₁₄₁ and C₁₆₅–G₁₇₁ (21–23)), additional nucleotides (the A₁₂₁–A₁₂₉ region) became protected upon interaction with Rex, suggesting the presence of multiple Rex binding sites. Investigation of the 5’-half of the 3'-RxRE with the primer RxRE P₂ did not reveal any GST-Rex-specific RNase protections (data not shown).
Probing the Secondary Structure of the 5'-RxRE—Although we found that Rex binds the 3'-RxRE and the 5'-RxRE equally well, it was uncertain whether the 5'-RxRE has the same secondary structure with respect to the important stem-loop D region. The 5'-RxRE does not contain the 33 nucleotides present in the 5'-end of the 3'-RxRE, where they take part in the formation of stem I and II. Consequently, the two RxREs must form different secondary structures, at least in the 5'-part of the molecules. To examine this, we made nuclease probing of the 5'-RxRE structure using RNases T1, T2, and V1 under the same conditions as for the 3'-RxRE (Fig. 4A). The result from three independent experiments is summarized in Fig. 4B. Most importantly, we find that the cleavage pattern of the 110–180 region is essentially identical in the two RxREs, which we interpret as the presence of a similar stem-loop D structure in both elements. From the cap site to position 50, the nuclease digestion pattern is also highly similar in the two RxREs (primary data not shown; compare Figs. 3B and 4B). In contrast, the regions 50–110 and 180–190 display significant differences between the two substrates. In particular, only in the 5'-RxRE were high reactivities of RNase V1 at positions C56, G57, and G68 and of RNase T1 at position G99 observed. Other positions within the 5'-RxRE showed diminished accessibility as compared with the 3'-RxRE, e.g. the T1 and V1 cleavages at positions G73 and U79, respectively. Combining the nuclease probing data with computer modeling (RNAdraw version 1.1 and RNA Fold) suggests a secondary structure of the 5'-RxRE as shown in Fig. 4B. Comparing this with the proposed structure of the 3'-RxRE (Fig. 3B) implies that the stem-loops A, B, and D are similar in the two RxREs, whereas a differently structured stem-loop C is formed. Finally, the stems I and II found in the 3'-RxRE obviously cannot be formed within the 5'-RxRE, since one of the participating RNA strands is missing.

The Rex Binding Site in the 5'-RxRE Overlaps the Major Splice Donor Site—The observations that the important stem-loop D of the 3'-RxRE is also present in the 5'-RxRE and that recombinant Rex binds the two RxREs equally well make it conceivable that Rex contacts the same positions in both RNAs. To test this possibility, we performed nuclease probing of His6-Rex binding to the 5'-RxRE using the same conditions as for...
FIG. 3. Ribonuclease probing of Rex binding to the 3′-RxRE. One pmol of 3′-RxRE RNA was incubated with 50 pmol of GST-Rex or the RNA binding mutant GST-Rex M1 with the same buffer conditions as in Fig. 2. The RNases T₁, T₂, A, and V₁ were used to analyze the RNA structure and positions in the RNA protected by Rex. Cleavages were monitored by primer extension using the primer RxRE P2 to assay the region from −33 to 80 and primer RxRE P1 to assay the region from 70 to 190. Since RNases T₁ and T₂ predominantly cleave single-stranded RNA and V₁ preferentially cuts in RNA helices, the cleavage pattern can be used to predict the secondary structure of the 3′-RxRE. A, autoradiogram of primer extension covering the region 100–190 of the 3′-RxRE. RNases and the presence of either wild-type GST-Rex (wt) or GST-Rex M1 (M1) are indicated above the lanes. A plus sign indicates control reactions treated as the nuclease samples but in the absence of RNase. The A, G, C, and U lanes refer to the RNA sequence and were generated by dideoxynucleotide sequencing of the untreated 3′-RxRE RNA template. Bars on the left denote protected regions. Nucleotides are numbered at the right from the beginning of the R region of the long terminal repeats. The overall lower intensity seen in lane 6 as compared with lane 5 was carefully normalized prior to quantifying the degree of protection. B, schematic representation of the RNase accessibility imposed on the secondary structure of the 3′-RxRE. The stem-loops A–D and the stems I and II are indicated. Observed cleavages are indicated by open circles (RNase V₁), squares (RNase T₁), and triangles (RNase T₂), respectively. The number of symbols at each nucleotide reflects the relative intensity of cleavages. The border between U3 and R (U3/R) and the 5′-splice site (5′SS) used in the 5′-RxRE are marked by large arrows, and the sequence complementary to the U1 small nuclear RNA at the 5′-splice site is shown in italic type. Data from RNase A treatment are not included, since they are inaccurate with respect to structural prediction. C, summary of Rex protections of the 3′-RxRE. Positions protected from RNase V₁ (open circles), RNase A (filled circles), RNase T₁ (squares), and RNase T₂ (triangles) cleavage are indicated, whereas unaffected digestions are excluded. +, + +, and + + + represent 1.5–2-, 2–3-, and >3-fold protection, respectively. Protection was quantified using an PhosphorImager as the intensity of cleavage in the presence of Rex M1 divided by the intensity of cleavage in the presence of Rex.
The 3'-RxRE (Fig. 4A). The result of three independent experiments is summarized in Fig. 4C. As can be seen, Rex protects essentially the same positions in the 5'-RxRE as in the 3'-RxRE, only with slight changes in intensities (compare Figs. 3C and 4C; the 5'-RxRE was not assayed with RNase A). Interestingly, in the 5'-RxRE the protected region overlaps the major splice donor site, which is located after G118, and which is used generating all spliced HTLV-I mRNAs. As with the 3'-RxRE, no effect on the RNase cleavage pattern upon Rex binding could be detected in the 5'-part of the 5'-RxRE using RxRE P2 as primer, even in the presence of a 200-fold molar excess of His$_6$-Rex to RxRE RNA (data not shown).

Modification Interference Analysis of the Interaction between Rex and the Stem-loop D—To substantiate the nuclease probing results, we investigated which nucleotides within the stem-loop D were critical to Rex binding by modification interference. 3'-end-labeled stem-loop D RNA was subjected to limited chemical modification with either A- and G-specific DEPC or C- and U-specific Hz/NaCl under denaturing conditions, gel-purified, and incubated with GST-Rex or His$_6$-Rex. The reactions were run on a nondenaturing polyacrylamide gel to separate unbound stem-loop D and stem-loop D-Rex complexes. The
conditions were chosen such that two complexes were formed, corresponding to complex A and B in Fig. 2D. The RNA was recovered from the gel and treated with aniline, which cleaves the RNA strand at modified residues. Comparing the intensities of cleavages of the unbound and the two complexed pools of RNAs shows which nucleotides cannot be modified without affecting the capacity to bind Rex (Fig. 5A). The differences in cleavages were quantified and are shown schematically in Fig. 5B for the GST-Rex experiment. In agreement with our RNase protection assays, modification of the positions G123, A128–C131, G135, and G136 reduced the affinity of the stem-loop D to GST-Rex. Moreover, positions U132–A142, A140–A142, C153, C156, and A170–A175, which were either not detectable or unaffected in the RNase protection analyses, were important to GST-Rex binding in this assay. Modification of certain nucleotides interfered strongly with formation of both complex A and B (Fig. 5B, closed symbols), whereas other positions showed stronger interference with B complex formation as compared with complex A (Fig. 5B, open symbols). The observed modification interferences are likely to reflect that GST-Rex is prevented from contacting the particular nucleotides, although we cannot rule out the possibility that some of the modifications disrupt an RNA structure important for GST-Rex binding. Modification interference experiments using His6-Rex gave similar results but with one noticeable difference (data not shown). Contrary to the negative interference observed at positions A140, A142, and C163 with regard to GST-Rex binding, DEPC-modification of G139–A142, as well as Hz/NaCl modification of C161–C163, caused enhanced His8-Rex binding. It is possible that modification of these residues reduces the stability of the helical structure formed by G139–G141 and C161–C163 and that this only affects GST-Rex, which binds the RxRE with a slightly lower binding affinity than does His8-Rex.

**DISCUSSION**

The repetitive nature of the termini of the HTLV-I genome implicates that part of the RxRE in the 3′-long terminal repeat is also present within the 5′-long terminal repeat. In this report, we show that HTLV-I Rex binds the RxRE structures derived from both ends of the viral transcript with equal specificity and affinity. The appearance of multiple complexes in the mobility shift assays with the two RxREs suggested the presence of more than one Rex binding site within each RxRE, and nuclease probing and modification interference studies revealed an extended Rex binding region as compared with previous reports.

The two RxREs have similarly structured stem-loops A, B, and D, as judged from our nuclease probing, whereas a different stem-loop C is formed in the two RNAs. The stem-loop D sequence has previously been shown to bind Rex in vitro (22), albeit with reduced affinity (24). Using gel mobility shift analysis, we found that the affinity is reduced approximately 30-fold as compared with the intact RxREs, suggesting that the overall structure of the RxRE is important for Rex association. This notion is also implicated by in vivo results showing that two tandem copies of the stem-loop D are required to confer Rex responsiveness (24). The absence of any protections against RNase cleavages in regions outside the stem-loop D, even in the presence of a large excess of Rex protein, suggests, however, that the adjacent structures primarily play an indirect role in Rex binding, probably through stabilization of the stem-loop D structure.

In accordance with the similarities in structure and Rex binding capacity, the ribonuclease probing of Rex binding to the 3′- and 5′-RtRE yielded similar protection patterns. Rex impeded cleavages in the regions encompassing A121–G136 and U167–G169, of which only the G130–G136 and U167–G169 regions...
have previously been observed to be critical to Rex binding in a modification interference study (22) and by in vitro selection (23). The A121–A129 region, which has not been demonstrated to be implicated in Rex binding earlier, was most strongly affected by Rex binding in our assay. Since the RNA footprinting was performed under conditions where at least two Rex molecules bind to the RxRE in a native gel, the extended footprint in the A121–A129 region most likely represent a second Rex binding site. This also may not have been detected in either the modification interference study by Bogerd et al. (22) (since only one complex was analyzed) or the in vitro selection experiment by Baskerville and co-workers (23), since not all nucleotides affected in our analyses were randomized, and therefore they could not be assayed. Despite titration of Rex in the ribonuclease protection experiments, we only found Rex-specific protection in the stem-loop D of the RxRE, indicating that only this region contains Rex binding sites. The multiple Rex molecules bound in the higher order complexes observed at the highest Rex concentrations in the mobility shift analyses, are, therefore, most likely either bound in this region or complexed merely through protein-protein interactions. The latter possibility is supported by the observation that Rex is able to form oligomers in the absence of RxRE (41).

The existence of at least two distinct Rex binding sites within the stem-loop D is substantiated by our modification interference analysis. Using the entire stem-loop D as a probe, we compared unbound RNA to RNA from first- and second-order complexes, denoted A and B, respectively. These experiments supported our finding of two separate domains composed of G129C172–U174 and G135–G136/G164–G165 that were equally important for formation of complex A. Nucleotides that displayed stronger interference with B complex formation as compared with complex A were mainly located between these regions (A128–A134 and A170–G171). Although we cannot exclude the possibility that some of the interference observed may occur at the level of RNA folding, the nuclease protection experiments suggest that both sites are occupied by Rex protein. Our data is most easily explained by the existence of at least two Rex binding sites within the stem-loop D, but they do not allow an exact determination of the number of Rex molecules in complexes A and B. The observed interference at two sites for complex A formation suggests that at least two Rex molecules bind cooperatively to multiple sites or that a single Rex molecule binds both sites in this complex. In agreement with this interpretation, we found that a minimal RNA, HB-13, containing only the distal binding site, binds Rex with about 5- and 100-fold lower affinity as compared with stem-loop D and RxRE, respectively. As indicated in Fig. 6, RNase protection and/or binding interference observed in this study coincide with two repeated structural motifs in the stem-loop D containing two G-C base pairs flanking a bulge that contains a uridine as the 5′-residue (boxed in Fig. 6). One motif located at the U137–C138 bulge was earlier demonstrated to be critical to Rex binding (22, 23), while we suggest that the second motif formed by the nucleotides G123, U124, G130, C172, and C173 constitutes a second Rex binding site not characterized previously. Between the two motifs, a similar motif is located (marked with a dotted box in Fig. 6). We have observed minor changes in RNase cleavage pattern upon Rex binding within this element as well; thus, it may represent a weaker additional Rex binding site. A secondary role for this bulge structure was suggested by Baskerville and co-workers (23) based on in vitro selection.

The importance of multiple Rex binding sites within the RxREs is reinforced by the previous observation, that Rex, when fused to a heterologous RNA binding domain, requires the presence of at least two copies of the corresponding target RNA sequence for minimal functionality, and a further induction is obtained using four copies (42). Moreover, alignment of 20 HTLV-I and three HTLV-II isolates and two isolates of the related simian T-cell leukemia virus type I shows no sequence variation with respect to the two putative Rex binding motifs, indicating a functional role of the nucleotides concerned (data not shown). The requirement of multiple binding sites resembles the functional necessity of HIV-1 Rev protein oligomerization on the RRE (37, 39). However, the two viral systems seem to differ at several important points. While only one localized binding site is detected for Rev in the RRE by modification interference analyses (35, 43), the RxREs appear to contain at least two distinct sites important for Rex binding. Moreover, only one copy of the RRE is present in the HIV-1 RNA genome, whereas two Rex binding stem-loop D structures most likely are formed in the full-length HTLV-I transcript. Finally, as a result of Rev oligomerization, the RRE harbors up to 10 Rev binding sites that can be detected by gel mobility shift analysis (39), while we only observe binding of about four Rex molecules within a limited subregion of the RxREs. Thus, in the context of viral replication, the presence of two RxREs could potentially compensate for the lower number of Rex molecules bound to each RxRE as compared with the Rev-RRE situation.

The Rex binding region in the 5′-RxRE overlaps with the consensus sequence of the 5′-splice site used for generating all spliced viral mRNAs (Fig. 6). This raises the possibility that the regulated expression of spliced and unspliced RNA species in part may depend on a direct regulation of splicing by Rex binding to the 5′-RxRE. Although considerable evidence favors a model in which Rex and the equivalent HIV-1 Rev protein facilitate nuclear export of unspliced and singly spliced viral RNA (7, 44, 45), the two mechanisms are not mutually exclusive. A direct function of Rex in splicing is supported by several observations. First, the 5′-splice signal in the 5′-RxRE is re-
ported to be essential to Rex responsiveness in vivo even in the absence of a downstream 3′-splice site (25). Second, Rex induces accumulation of unspliced viral RNA in the nucleus as well as in the cytoplasm of T-cells in an RxRE-dependent manner, concurrently with a decrease in the amount of spliced viral RNA (14). Third, the very homologous HTLV-II Rex protein can in vitro specifically inhibit an early splicing step of a pre-mRNA consisting of the HTLV-II 5′-RxRE positioned upstream of an adenovirus-derived 3′-splice site (27). Since the recognition of the 5′-splice site by splicing factors is an important step in committing pre-mRNA to splicing, it is possible that binding of Rex to the 5′-RxRE interferes with the recognition of the downstream RNA as an intron. Consequently, full-length RNA escapes nuclear retention and is exported to the cytoplasm, mediated by the nuclear export signal of Rex.

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