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The Rev-Responsive Element Negatively Regulates Human Immunodeficiency Virus Type 1 *env* mRNA Expression in Primate Cells

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The human immunodeficiency virus type 1 (HIV-1) Rev protein mediates the accumulation of unspliced and singly spliced viral transcripts within the cytoplasm of infected cells, late in the infection cycle, leading to the expression of the viral structural proteins, Gag, Pol, and Env. Rev binds to a complex RNA structure, the Rev-responsive element (RRE), present in all Rev-responsive viral transcripts, relieving their nuclear sequestration. The precise mechanism by which RRE-containing transcripts are retained within the nucleus in the absence of Rev protein is not well understood. We previously demonstrated that the RRE alone plays a crucial role in the nuclear retention of RRE-containing *env* transcripts in stably transfected *Drosophila* cells. Here we extend our previous observations and demonstrate that the RRE is a principal determinant of nuclear retention for envelope transcripts in primate cells and, in particular, human CD4$^+$ T cells.

The human immunodeficiency virus type 1 (HIV-1) encodes a number of regulatory and accessory proteins in addition to the characteristic *gag*, *pol*, and *env* genes common to all members of the retroviral family. Analysis of RNA from HIV-1 infected cells demonstrates that the various gene products are expressed from a set of HIV-specific mRNAs that are produced by alternative splicing of a single-genome-length primary viral transcript (15, 19, 27). These mRNAs fall into two subsets: the small multiply spliced mRNAs that encode the regulatory proteins, Tat, Rev, and Nef, and the singly and unspliced mRNAs that encode the structural proteins, Gag, Pol, and Env.

Kinetic studies reveal that the multiply spliced mRNA species appear in the cytoplasm of infected cells early in the infection cycle whereas the singly spliced and unspliced viral transcripts are retained within the nucleus. Subsequently, late in infection, the incompletely spliced mRNAs are also released from the nucleus, allowing for expression of the viral structural proteins (27). This transition in the pattern of the viral transcripts appearing in the cytoplasm is dependent on the viral regulatory factor, Rev (for reviews, see references 9, 14, 16, 30, 31, 38, and 40).

The 116-amino-acid phosphoprotein, Rev, localizes to the nucleus of expressing cells (10) and binds to a region of complex RNA structure, the Rev-responsive element (RRE), present in the *env* regions of all singly and unspliced viral transcripts (11, 12, 20, 32, 36, 42, 43). A growing body of information suggests that the Rev-RRE interaction activates these incompletely spliced transcripts for nuclear export through an active transport pathway, resulting in efficient expression of the viral structural proteins (3, 17, 18, 39). However, the precise mechanism by which RRE-containing transcripts are retained within the nucleus of infected cells, in the absence of Rev, remains unclear. Independently proposed models suggest that recognition of splice site information by splicing components, or various repressive sequences scattered throughout the viral genome, are responsible for the observed nuclear retention of incompletely spliced viral transcripts (6, 8, 28, 29, 34, 36).

Our recent efforts have centered on the use of stably transfected *Drosophila melanogaster* S2 cells for dissection of the Rev-regulatory pathway (4, 26, 33). Using the *Drosophila* system, we were able to recapitulate the Rev-dependent expression of the HIV-1 envelope glycoprotein that is normally observed in transfected mammalian cells, including human CD4$^+$ T cells. In *Drosophila* cells, gp160 envelope protein is not expressed in the absence of Rev, but the block to expression can be overcome by supplying Rev in *trans*. Most importantly, in the absence of Rev, the RRE-containing *env* transcripts are retained within the nuclear compartment of transfected *Drosophila* cells, and only when Rev is coexpressed do they locate to and accumulate within the cytoplasm, resulting in expression of envelope (4, 26). Thus, the efficient nuclear retention of *env* transcripts in Rev-deficient *Drosophila* cells suggests the existence of a common mechanism of repression that is maintained in both *Drosophila* and human cells.

Our analysis in the *Drosophila* system indicated that splice sites are not required for nuclear retention of *env* transcripts. Instead, we found that a single, *cis*-acting repressive sequence (CRS) element was both necessary and sufficient for nuclear retention and Rev responsiveness. This CRS element mapped within the RRE region (4). All constructs in which the RRE region was removed or substantially altered were no longer nuclear retained but rather were efficiently transported to the cytoplasm and expressed in the absence of Rev. The data implicated the RRE as a key element in nuclear retention as well as in Rev responsiveness of *env* mRNAs.

It has been argued that the *Drosophila* system is a special case and does not reflect the relevant mechanisms underlying the Rev response in mammalian cells. Hence, in this report we attempt to address the issues raised by our previous data and specifically examine the features that are required for nuclear retention and Rev responsiveness of *env* transcripts in monkey and human cell lines.

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We demonstrate that the RRE acts as a primary determinant of nuclear retention in transiently transfected heterologous mammalian cell systems and, most importantly, in human $CD4^+$ T cells. Our results confirm the importance of the RRE as a critical element in the nuclear retention of *env* transcripts and highlight the utility and predictive value of the *Drosophila* system for analysis and dissection of the Rev-regulatory pathway. Clearly, a common mechanism for nuclear retention of *env* mRNAs exists in both insect and mammalian cell types. In HIV infection, both unspliced (*gag-pol*) and singly spliced (*env*) mRNAs are subject to nuclear retention and positive regulation by Rev. Our results implicate a common region to all of these transcripts, the RRE, as perhaps the only CRS required for achieving nuclear retention.

MATERIALS AND METHODS

Plasmids. Vectors pMC160, pMC120, pMC160Δ3′, and pMC160Δ3′ΔRRE were derived from the previously described vectors pMt160Δ32, pMt120Δ32, pDB160ΔSA, and pDBΔRRE, respectively (4, 26). The entire *env* transcription unit of each vector, spanning from 25 bp upstream of the ribosome binding site and extending through the simian virus 40 early polyadenylation sequence, was subcloned into the mammalian expression vector pCDN (1). The resulting vectors contain previously described derivatives of the *env* gene under the control of the cytomegalovirus promoter and the simian virus 40 early polyadenylation signal (1, 4). The vector pRSV-Rev (25) contains the Rev coding sequence, the Rous sarcoma virus promoter, and HIV-1 polyadenylation signals in pUC18.

Cell culture. The HeLa and Cos-1 cell lines, obtained from the American Type Culture Collection, were maintained in Dulbecco modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 100 U each of penicillin and streptomycin per ml of medium. Jurkat cells were obtained from the American Type Culture Collection and maintained in RPM 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 100 U each of penicillin and streptomycin per ml of medium.

Transfections. Cos and HeLa cells were transfected using Lipofectamine (Gibco, BRL) according to the manufacturer's instructions. Jurkat cells were electroporated by using a Bio-Rad Gene Pulser according to the manufacturer's instructions. Transfection mixtures were incubated for 48 h and 72 h, at which time samples were harvested for protein and RNA analyses, respectively.

RNA isolation and analysis. Total and cytoplasmic poly(A)$^+$ RNA samples were isolated by using an oligo(dT) magnetic bead procedure as directed by the manufacturer [Dynabeads Oligo$(dT)_{25}$; Dynal]). Total and cytoplasmic poly(A)$^+$ RNA samples equivalent to 2.0×10^6 cells were resolved on 1% agarose gels containing formaldehyde and transferred to nitrocellulose membranes (4). To detect *env* mRNA, blots were blocked and probed with a ^{32}P-labeled random-primed *Stu*I-*Hin*dIII *env* fragment (4). Blots were washed and exposed to X-ray film. To ensure equivalent loading, blots were stripped by boiling for 15 min in 0.1% sodium dodecyl sulfate (SDS)–0.1 mM EDTA and reprobed with a labeled fragment derived from the human glucose-6-phosphate dehydrogenase gene.

Protein analysis and Western blotting (immunoblotting). Proteins were resolved on SDS-polyacrylamide (10 or 12%) gels and electrophoretically transferred to nitrocellulose (Schleicher & Schuell) as described previously (4, 5). Blots were probed by using 1:3,000 or 1:6,000 dilutions of primary antisera, and proteins were detected by enhanced chemiluminescence as directed by the manufacturer (Amersham). Envelope glycoprotein expression from Jurkat cells was monitored by immunoprecipitation of gp120 from culture supernatants, using the anti-gp120 monoclonal antibody 178.1 (41), followed by Western blotting of the precipitated material.

RESULTS

Splice site-independent Rev responsiveness of envelope glycoprotein expression in Cos-1 cells. Previously, we had developed a number of envelope expression vector constructs which when stably transfected into *Drosophila* cells demonstrated that both nuclear retention and subsequent Rev-dependent expression of envelope were dependent on the integrity of the RRE and did not require any splice site information. In an effort to examine whether these results were specific to the *Drosophila* system used or, alternatively, reflected the general intrinsic mechanism of Rev regulation, we carried out a set of analogous experiments using mammalian cells. Each of the previously described *env* transcription units (4, 26) was excised from the *Drosophila* expression vectors and incorporated into the mammalian expression vector pCDN (1), placing the *env* region under the control of the cytomegalovirus promoter. The various transcription units are otherwise identical to those used in the *Drosophila* experiments, including sequences spanning from 25 bp upstream of the ribosome binding site and extending through the entire *env* coding region and the simian virus 40 early polyadenylation site. The envelope constructs are slightly chimeric at the 5′ end. All upstream sequences, including the envelope leader and signal sequence and the splice donor for the *tat* and *rev* RNAs, have been removed and replaced by the signal sequence of the tissue plasminogen activator gene. The remainder of *env*, and in particular the RRE, was maintained in order to avoid disruption of the complex RNA structure which we suspect plays a role in the regulation. The various *env* expression constructs generated are shown in Fig. 1.

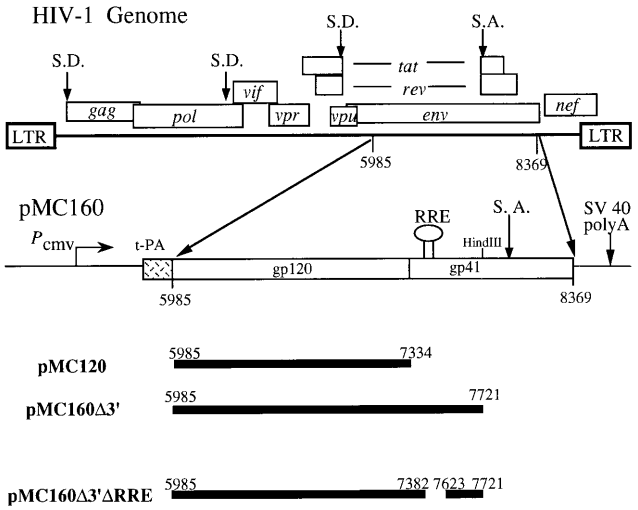

FIG. 1. The HIV-1 genome and plasmid constructs. The major genes and splice donor (S.D.) and splice acceptor (S.A.) sites are depicted. LTR, long terminal repeat; P_{cmv}, cytomegalovirus promoter; t-PA, signal sequence from the tissue plasminogen activator; SV 40 polyA, simian virus 40 early polyadenylation signal; open rectangles, *env* sequences; thin line, vector sequences. The solid black bars indicate the regions of *env* present in each construct, with nucleotide coordinates shown (according to reference 35).

To test the Rev dependence of these *env* constructs in mammalian cells, each expression vector was transiently transfected into Cos-1 primate cells in the presence or absence of the Rev expression vector, pRSV-Rev (25). Western blot analysis of cell-free conditioned medium from these transfected cell lines was used to monitor the expression of envelope proteins from the various constructs (Fig. 2).

We found that the parental vector, pMC160, encoding the full-length gp160 glycoprotein, expressed envelope protein in a highly Rev-dependent manner, as indicated by the appearance of the mature gp120 glycoprotein in the culture supernatant only in the presence of Rev (Fig. 2, lane 2). The envelope transcription unit in pMC160 lacks the natural 5′ Tat/Rev splice donor site (which has been deleted in all of our constructs) but retains the 3′ Tat/Rev splice acceptor site, thereby indicating that the 5′ splice donor is not required for Rev regulation in primate cells (37).

In an analogous experiment, a derivative construct, pMC160Δ3′, which lacked both the Tat/Rev splice donor and acceptor sites as well as the distal coding region of gp41, was found also to be fully Rev dependent. No envelope protein was expressed in the absence of Rev, but the block to expression was relieved by supplying Rev in *trans* (Fig. 2, lanes 5 and 6). Thus, neither splice site is required for Rev dependence in this

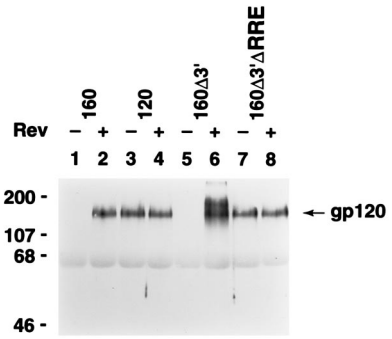

FIG. 2. Rev responsiveness of envelope glycoprotein expression is independent of splice sites in Cos-1 cells. Cos-1 cells transfected with pMC160, pMC120, pMC160Δ3′, or pMC160Δ3′ΔRRE in the absence (−) or presence (+) of pRSV-Rev were assayed for envelope protein expression (gp120) by Western analysis of culture supernatants, using anti-gp120 polyclonal antisera. gp120 is indicated by the arrow; the upper band produced from pMC160Δ3′ and pMC160Δ3′ΔRRE corresponds to unprocessed gp160 which occurs as a result of the deletions in the gp41 region of *env* (4). Sizes are indicated in kilodaltons.

primate cell line. Furthermore, the sequences deleted in this vector from the terminal coding region of gp41 are also not involved in Rev regulation.

In dramatic contrast to the Rev-dependent expression observed with the constructs described above, two other vectors, pMC120 and pMC160Δ3′ΔRRE, which lack additional sequences within the gp41 coding region of *env*, expressed envelope protein independently of Rev (Fig. 2, lanes 3 and 7, respectively). Significantly, both of these *env* expression constructs retain all of the gp120 coding sequence but contain deletions within the gp41 coding region that remove the RRE. pMC120 is missing the entire gp41 coding region, whereas pMC160Δ3′ΔRRE contains a precise 240-nucleotide in-frame deletion of RRE sequences but is otherwise identical to the Rev-dependent construct pMC160Δ3′. Thus, deletion of the RRE region appears to be directly responsible for the Rev-independent expression of envelope in Cos cells. Furthermore, these data indicate that no other inhibitory sequences (i.e., CRS elements) exist within the gp120 coding region of *env* that are recognized by this primate cell line. In this respect, it is important to note that both pMC120 and pMC160Δ3′ retain the minor tev splice site (2, 22) present within the gp120 coding region of our *env* constructs. Nevertheless, this site is unable to prevent Rev-independent envelope expression. Thus, cryptic splice sites present within the transcription unit are not relevant to Rev regulation of envelope expression.

Recognition of the CRS element within the RRE is conserved across mammalian cell types. The data presented above indicate that the Tat/Rev splice sites are not required for Rev regulation of *env* transcripts in the monkey kidney cell line Cos-1. Instead, a single inhibitory sequence responsible for the observed Rev dependence occurs within the gp41 coding region, within or overlapping the RRE. In an effort to examine whether the pattern of Rev regulation observed in the Cos cell line was applicable more generally in human cells, we carried out a series of transient transfections in the human HeLa cell line, using the same vector constructs.

Western blot analysis of culture supernatants from transiently transfected HeLa cells (Fig. 3A) indicated that the pattern of Rev dependence observed for envelope glycoprotein expression was essentially identical to that observed in Cos cells. In HeLa cells, the *env* construct pMC160 (Fig. 3A), encoding the full-length gp160, expressed gp120 envelope glycoprotein in a Rev-dependent manner (lanes 1 and 2). Likewise, construct pMC160Δ3′, which lacks both of the major Tat/Rev splice sites, was also Rev dependent, indicating yet again that these splice sites are not required for the repressive effects observed in HeLa cells (lanes 5 and 6).

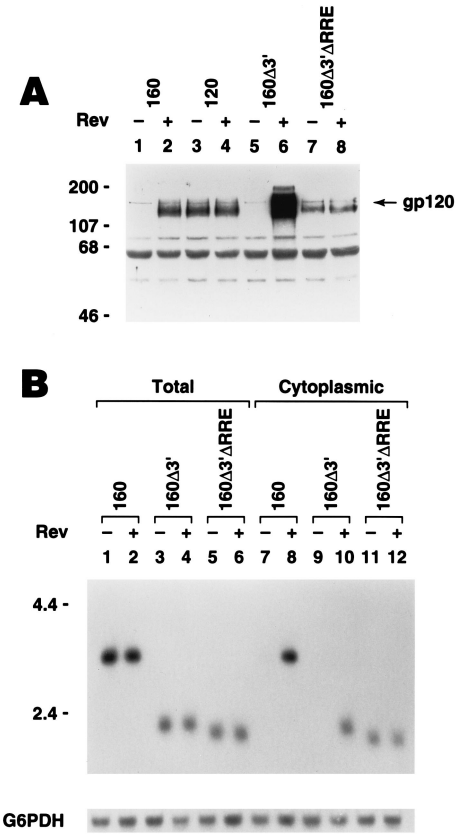

FIG. 3. The RRE is responsible for nuclear retention of *env* mRNAs from human cells. (A) HeLa cells transfected with pMC160, pMC120, pMC160Δ3′, or pMC160Δ3′ΔRRE in the absence (−) or presence (+) of pRSV-Rev were assayed for envelope protein expression (gp120) by Western analysis of culture supernatants, using anti-gp120 polyclonal antisera; gp120 is indicated by the arrow. Sizes are indicated in kilodaltons. (B) Northern blot of total and cytoplasmic RNA fractions from HeLa cells transfected as described above. Expected *env* transcript sizes are as follows: pMC160, 3 kb; pMC160Δ3′, 2.4 kb; and pMC160Δ3′ΔRRE, 2.2 kb. The blot was reprobed with a fragment of the human glucose-6-phosphate dehydrogenase (G6PDH) gene as a control for equal loading.

Analogous to the results obtained in Cos cells, we found that expression in HeLa cells from constructs lacking RRE sequences did not require Rev. Both pMC120, which lacks all gp41 coding sequences, and pMC160Δ3′ΔRRE, which lacks the RRE region but is otherwise identical to pMC160Δ3′, expressed envelope protein in both the presence and absence of Rev (Fig. 3A, lanes 3, 4, 7, and 8). Again, deletions that specifically remove RRE sequences from *env* overcome the block to envelope expression and relieve the requirement for Rev. Thus, the results obtained from the human HeLa cell line demonstrate a consistent pattern of Rev responsiveness from insect to human cells.

The RRE is responsible for the nuclear retention of *env* mRNAs in human cells. By analogy to results previously obtained from the *Drosophila* system, we suspected that the block to *env* expression in primate and human cells was due to the RRE-mediated nuclear retention of *env* transcripts. To test this view, we examined the subcellular distribution of *env* mRNAs in the transfected HeLa cells. Northern blot analysis

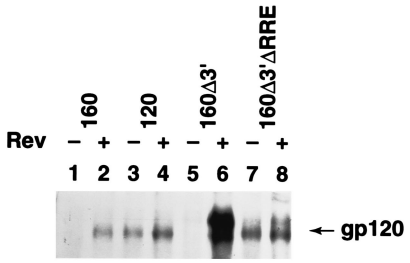

FIG. 4. Within *env*, the RRE is the primary inhibitory element recognized in $CD4^+$ T cells. Jurkat cells transfected with pMC160, pMC120, pMC160Δ3′, or pMC160Δ3′ΔRRE in the absence (−) or presence (+) of pRSV-Rev were assayed for envelope protein expression (gp120). gp120 was immunoprecipitated with monoclonal antibody 178.1 (41) from culture supernatants, and the precipitated material was analyzed by Western blotting as described in the text.

demonstrated that transcripts from the RRE-containing *env* constructs pMC160 and pMC160Δ3′ accumulated within the cytoplasm of transfected cells only in the presence of Rev (Fig. 3B, lanes 8 and 10, respectively). In Rev-deficient cells, these RRE-containing transcripts were retained within the nucleus and failed to accumulate within the cytoplasmic compartment (Fig. 3B, lanes 7 and 9, respectively).

A different pattern of envelope mRNA expression was observed with constructs that lacked RRE sequences. We found that mRNA from the pMC160Δ3′ΔRRE construct accumulated efficiently within the cytoplasm of transfected cells in the absence and presence of Rev protein (Fig. 3B, lanes 11 and 12). Identical results were obtained with pMC120 (data not shown). Thus, deletion of 240 bp of sequence overlapping the RRE reverses the nuclear sequestration of *env* transcripts in human cells (compare pMC160Δ3′ and pMC160Δ3′ΔRRE in Fig. 3B, lanes 9 and 11). It is also important to note that these transcripts undergo no detectable splicing (Fig. 3B).

Within *env*, the RRE is the primary inhibitory element recognized by $CD4^+$ T cells. All of the data which we have presented to date were obtained from a variety of cell types that are not normally infected by HIV-1. However, it is likely that the RRE plays an identical role in $CD4^+$ human T cells, one of the normal cell targets of HIV-1 infection. To test this, we carried out a series of transient transfections into the $CD4^+$ Jurkat T-cell line, using the same set of *env* expression vectors as described above.

Because of the low transient transfection efficiencies obtained with Jurkat cells, envelope expression was monitored by direct immunoprecipitation of gp120 from culture supernatants followed by Western blotting of the precipitated materials. The results (Fig. 4) demonstrate the same overall pattern of Rev responsiveness as observed with the other cell types. Both pMC160 and pMC160Δ3′, which contain an intact RRE, were strongly Rev dependent for envelope glycoprotein expression (Fig. 4, lanes 1, 2, 5, and 6). In contrast, cells transfected with the RRE-deficient construct pMC160Δ3′ΔRRE or pMC120 constitutively expressed envelope glycoproteins in either the presence or absence of Rev (Fig. 4, lanes 3, 4, 7, and 8). Thus, in human $CD4^+$ T cells, deletion of RRE sequences appears to be all that is required to allow Rev-independent expression of Env protein.

DISCUSSION

We have demonstrated that the RRE plays a critical role in the nuclear retention as well as the Rev responsiveness of RRE-containing *env* transcripts in various primate cells, including human T cells. Combined with our earlier observations in *Drosophila* cells, these results suggest that the intrinsic mechanism of action of Rev regulation may be common to both insect and mammalian cells. Specifically, in the context described herein, we have shown (i) that Rev regulation of envelope glycoprotein expression occurs on transcription units that retain an intact RRE sequence but lack the major Tat/Rev splice donor and acceptor sites and that undergo no detectable splicing; (ii) that the presence of at least one minor splice site (tev) is unable to repress *env* expression from constructs that lack RRE sequences; (iii) that sequences within the RRE are directly responsible for the repression of envelope expression; and (iv) that the inhibition of envelope expression is due directly to the RRE-mediated nuclear retention of *env* transcripts. Thus, the results presented here establish the importance of a single CRS located within the RRE as a principal determinant of nuclear retention and Rev responsiveness for *env* transcripts in both insect and mammalian cell systems. Importantly, this negative element is recognized and is fully functional in human $CD4^+$ T cells.

Chang and Sharp (6) have suggested that HIV-1 splice sites are responsible for the nuclear entrapment of RRE-containing mRNAs. Our data do not support that conclusion; the negative regulation by nuclear retention required to support Rev responsiveness can be achieved readily in multiple cell types on the *env* transcripts described herein which lack all major HIV-1 splice sites. Furthermore, the presence of cryptic splice sites (e.g., tev sites) does not contribute to nuclear retention of mRNAs that lack functional RRE sequences; these mRNAs readily accumulate within the cytoplasm of transfected cells in the absence of Rev.

Other reports have also questioned the requirement for splice site information (14, 16, 18, 30, 34, 36). Using *Xenopus laevis* oocytes, Fischer et al. (18) demonstrated that an RRE-containing mRNA molecule that lacked intron sequences was retained within and exported from the nucleus in a Rev-dependent manner. Furthermore, an RRE-containing intron excised as a closed lariat structure also was retained within the nucleus and transported to the cytoplasm in a Rev-dependent fashion. In addition, Nasioulas et al. (34), using a mammalian cell system, demonstrated that following removal of all known splicing information, *env* mRNA remained Rev responsive when it contained a functional RRE. Those results are consistent with those presented here. However, Nasioulas et al. also found a repressive element within the gp120 coding region. In contrast, we find no evidence for alternative repressive sequence elements, and we suggest that the RRE may be all that is required to support both the negative and positive aspects of the Rev response.

Our results have significant implications regarding the intrinsic mechanism of Rev function. The data presented, originally from the *Drosophila* system and now from human and monkey cells, indicate that Rev protein, in binding to the RRE, binds at or close to a negative regulatory signal that it suppresses. It is well known that the structure of the RRE plays an important role in the positive aspects of the Rev response through its capacity to bind the Rev *trans* activator (7, 13, 23, 24). We suggest that the complex RRE structure also is important for those negative aspects of the Rev response that are associated with the nuclear retention of RRE-containing transcripts (33). In fact, several investigators have noted that certain RRE-containing transcription units fail to be negatively regulated (i.e., retained in the nucleus) (6, 20–22). They have interpreted their results as suggesting that the RRE plays no role in negative regulation. One possibility uniformly overlooked by these investigators is that in the specific context of

their chimeric constructs, the structural integrity of the RRE required for its negative effects has been disrupted. Recent evidence from our own laboratory (33) indicates that the structural integrity of the RRE is essential for its negative regulatory role in Rev regulation of envelope transcripts. Thus, the RRE structure may be all that is required for achieving the inhibitory events associated with nuclear retention. If cellular factors are also involved, they will have to be evolutionarily highly conserved proteins.

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