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# Rev of human immunodeficiency virus and Rex of the human T-cell leukemia virus type I can counteract an mRNA downregulatory element of the transferrin receptor mRNA

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## ABSTRACT

**Expression of the structural proteins of the human immunodeficiency virus type 1 (HIV-1), the human T-cell leukemia virus type I (HTLV-I), and of the transferrin receptor (TfR) mRNA depends on posttranscriptional regulatory mechanisms involving both positive and negative elements. In these systems the presence of elements decreasing mRNA expression have been demonstrated. The regulatory proteins (Rev, Rex or iron response element binding protein IRE-BP) antagonize the effects of the downregulatory elements by interacting directly with specific mRNA sites (Rev responsive element, RRE, Rex responsive element, RXRE, or iron responsive elements, IREs) resulting in stabilization and efficient expression of the corresponding mRNAs. To investigate whether this strategy involves common pathways of mRNA utilization, we have studied expression from hybrid mRNAs that contained these previously identified HIV-1 or TfR instability determinants and the binding sites of the regulatory proteins Rev, Rex and/or IRE-BP. Our results demonstrate that only low levels of these hybrid mRNAs accumulate in the absence of the positive regulatory factors Rev, Rex or IRE-BP. The presence of these factors counteracts the effect of heterologous downregulatory elements resulting in increased accumulation of the hybrid mRNAs. However, while Rev or Rex regulation also resulted in efficient protein expression, the IRE-BP only affected mRNA levels without significantly affecting protein expression, suggesting that the pathways of mRNA stabilization/ expression are different in these systems.**

## INTRODUCTION

A subset of the HIV and HTLV mRNAs (for reviews see (1, 2, 3, 4, 5, 6, 7, 8, 9) and references therein) as well as many cellular mRNAs (i.e. TfR, c-myc, c-fos, GM-CSF, IL-2,  $\beta$ -interferon, for reviews see (10, 11)) use complex posttranscriptional steps to achieve regulated expression. These mRNAs are expressed at low levels due to the presence of negative elements presumably interacting with cellular destabilization factor(s). To achieve expression of these mRNAs, the presence of specific positive regulators is necessary. The complex regulation by positive and negative acting factors found in the HIV and HTLV viruses and cellular mRNAs is thought to be important and possibly allows temporary control of expression.

Both HIV-1 and HTLV-I viruses use an elaborate post-transcriptional regulatory system which involves both nuclear and cytoplasmic events to control expression of the viral structural proteins (see above for references). The expression of the *gag/pol* and *env* mRNAs of HIV-1 and HTLV-I depends on the regulatory proteins Rev and Rex, respectively. This subset of viral mRNAs is defective in stability, transport to the cytoplasm, and polysomal loading. These downregulatory properties are counteracted by the interaction of Rev or Rex with distinct elements located on the viral mRNAs termed Rev responsive element (RRE) and Rex responsive element (RXRE), respectively. Rev has been shown to shuttle from the nucleus to the cytoplasm (12, 13). These findings support the model that Rev chaperones the RRE-containing subset of viral mRNAs from the nucleus to the cytoplasm. Another model for Rev function proposes that the presence of inefficiently used splice sites on the viral mRNA is essential for Rev to exert its function (14, 15). Accordingly, poor expression of viral mRNAs is caused by inefficient splicing and

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the retention of these mRNAs in the nucleus in the absence of Rev.

We and others have identified multiple RNA elements located on these viral mRNAs that are responsible for their low expression in the absence of the viral regulatory proteins (16, 17, 18, 19, 20). One well-studied element partially responsible for this low expression is located within the p17<sup>gag</sup> gene of HIV-1 and was named INS-1 (16). We demonstrated that INS-1 affects the mRNA half-life when present in *cis*. We have inactivated INS-1 by introducing multiple point mutations maintaining an intact coding region of p17<sup>gag</sup> (17). This resulted in high level Rev-independent expression of p17<sup>gag</sup>.

The regulation of the Rev- and Rex-dependent mRNAs has some similarities to the iron-regulated expression of the TfR mRNA (for a review, see (21)). The 3' untranslated region of TfR mRNA contains a region encompassing five homologous hairpin structures termed iron response elements (IREs) and an element conferring RNA instability. This region, here termed IRR (Iron Response Region) is sufficient to render an otherwise stable mRNA unstable and regulatable by iron (22). The regulatory protein IRE-BP (Iron Response Element Binding Protein) binds to the IRE hairpins within IRR and counteracts the effect of the mRNA destabilization element. This leads to stabilization and efficient expression of TfR mRNA. The IRE-BP RNA-binding activity depends on the iron concentration. Under conditions that limit iron levels, the activated IRE-BP binds to the IREs resulting in increased TfR expression. The availability of iron leads to inactivation of the IRE-BP. Inactive IRE-BP cannot bind to the IREs, which results in unstable TfR mRNA.

To further understand the posttranscriptional regulation, we investigated whether a regulatory factor such as the IRE-BP can counteract an HIV-1 downregulatory element; and vice versa, whether positive regulators of HIV-1 and HTLV-I (Rev and Rex, respectively) can counteract a downregulatory element found in TfR mRNA. Hybrid mRNAs were studied that contain the instability elements INS-1 of HIV-1 and/or of TfR mRNA in addition to the targets for positive regulators such as Rev, Rex and/or IRE-BP. Here we show that the functionally analogous components of the HIV-1 (or HTLV-I) and TfR regulatory systems are partially interchangeable. This study demonstrates for the first time that the Rev-RRE as well as Rex-RXRE interaction can overcome the effect of a cellular downregulatory element.

## MATERIALS AND METHODS

### Recombinant plasmids

p17 contains HIV-1 LTR promoter, the HIV-1 p17<sup>gag</sup> matrix gene followed by the HIV-1 3' LTR providing the polyadenylation signal (nt 8561–9265 following the numbering of HXB2R (23)). p17R contains a complete RRE element as a 330-bp StyI fragment (24) downstream of the p17<sup>gag</sup> gene, followed by the HIV-1 3' LTR (nt 8021–9265). p17M (formerly called pNL17M1234 (17)) is analogous to p17 but contains multiple point mutations within the p17<sup>gag</sup> gene, which destroy the identified inhibitory/instability element (INS-1). p17.I and p17M.I, resulted from insertion of a 250 bp XbaI–BamHI fragment of pTRS-1 (22) bearing a minimal synthetic analog of IRR into the EcoRI–Asp718 sites of p17 and p17M, respectively. p17R.I was constructed by insertion of the IRR into the BamHI site of p17R. p17MR.I contains the complete RRE of HIV-1 inserted into the XbaI site of p17M.I. p17MR contains an

EcoRI–BamHI RRE fragment from the intermediate plasmid pUS330 inserted in the EcoRI–Asp718 of p17M. pUS330 was constructed by inserting the 330-bp StyI RRE-containing fragment (24) in the SmaI site of pUC19. p17MX contains the HTLV-I RXRE element. RXRE was excised as a 300-bp BamHI–Asp718 fragment from pBS-RXRE (25) and was inserted in the EcoRI–Asp718 sites of p17M. p17MX.I contains both the HTLV-I RXRE and the IRR elements. To construct this plasmid the IRR was excised from pTRS-1 as XbaI–BamHI fragment and cloned into the XbaI–BamHI of pBS-RXRE resulting in pBS-RXRE-TRS. Then the RXRE and IRR elements were cloned as a 530-bp XbaI–Asp718 fragment in the EcoRI–Asp718 of 17M, resulting in p17MX.I.

### Cells and transfections

HLtat cells is a HeLa-derived cell line that constitutively expresses HIV-1 Tat protein (26). RD4 is a human rhabdomyosarcoma cell line. HLtat and RD4 cells were seeded at concentrations of  $7 \times 10^5$  and  $1 \times 10^6$  per 60 mm plate, respectively. The next day the cells were transfected by the calcium phosphate coprecipitation technique as described (27, 28) using plasmid DNA purified on QIAGEN columns, according to the manufacturers instructions. The cells were incubated with the precipitates for 4 h (HLtat) or 2 h (RD4). Transfections into RD4 cells also included HIV-1 *tat* expression plasmid, pL3tat (26, 29). To study the Rev or Rex response, the transfection mixtures contained 1  $\mu$ g of the Rev-expressing plasmids pLsRev or pCMVsrev (30) or 2  $\mu$ g pRSVRex (24), respectively. All transfections included the luciferase expression plasmid pRSVluc (31) and subsequent measurements of luciferase activity in cell lysates were performed to control for transfection efficiency. Similar amounts of luciferase were produced within each experiment. RD4 cells were treated overnight with 10  $\mu$ M hemin or 100  $\mu$ M desferrioxamine. Hemin and desferrioxamine were prepared as 2 mM and 10 mM stock solutions, respectively, and were added to the culture medium immediately before use. One day post-transfection total RNA and protein were isolated from duplicate plates as described (32, 33). Nuclear and cytoplasmic mRNAs were prepared as described (34).

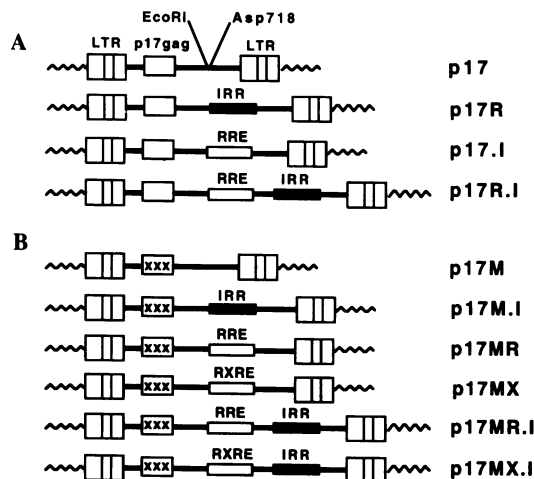
### Analyses of mRNA and protein produced from transfected cells

20  $\mu$ g RNA was subjected to Northern blot analysis as described (33). HIV-1 mRNAs were detected with a probe spanning nt 8304–9008 of the molecular clone HXB2. TfR mRNAs were detected using a 2.6 kb EcoRV–BamHI fragment of pTRS-1 (22).  $\beta$ -actin mRNAs were detected using a 2 kb HindIII fragment of  $\beta$ 2000 (35) as a probe. HIV-1 p17<sup>gag</sup> protein was visualized on Western blots as described (33) using HIV-1 patient plasma (Scripps Laboratory, Inc.). Blots were quantitated by scanning the autoradiographs in Ultrascan XL densitometer or by the AMBIS radioanalytic imaging system.

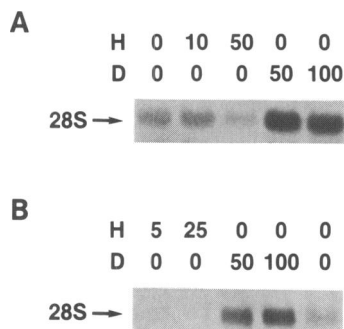
## RESULTS

### Regulation by IRE-BP and Rev in RD4 and HLtat cell lines

Regulation of TfR mRNA has been studied mostly in murine tissue culture systems. Since it was found that Rev functions poorly in murine cells (our unpublished data) or it does not function at all (36), we first identified human cell lines that allowed both IRE-BP and Rev regulation. We studied the



**Figure 1.** Schematic structures of the hybrid constructs. All plasmids contain the HIV-1 5' LTR as promoter and the HIV-1 3' LTR providing the polyadenylation signal. The p17 series (panel A) contains HIV-1 wild-type p17<sup>gag</sup> coding sequences bearing the instability element INS-1. In the p17M series (panel B) the INS-1 element within the p17<sup>gag</sup> gene is inactivated by point mutations indicated by XXX (see Materials and Methods). RRE (R) represents the Rev responsive element of HIV-1; RXRE (X) represents the Rex responsive element of HTLV-I; IRR (I) represents the iron response region of the transferrin receptor. The location of the major cloning sites (EcoRI and Asp718) is indicated.



**Figure 2.** Regulation of endogenous TfR mRNA in HLtat and RD4 cell lines. HLtat (A) and RD4 cells (B) were treated overnight with hemin (H) or desferrioxamine (D), using the indicated concentrations (in  $\mu\text{M}$ ). Total RNA was extracted and subjected to Northern blot analysis. The blots were hybridized with a 2.6-kb EcoRV–BamHI fragment of pTRS-1 (22), which contains the TfR structural gene and IRE element. Radioactivity in the bands was quantitated by the AMBIS radioanalytic imaging system. The position of 28S rRNA is indicated. Different exposure times are shown for panels (A) and (B). While TfR mRNA levels are readily detectable in hemin treated HLtat cells, they are not detectable in RD4 cells even in a longer exposure.

regulation of endogenous TfR mRNA by IRE-BP in RD4 and HLtat cells. RD4 is a human rhabdomyosarcoma cell line and was previously used to study TfR regulation (37). HLtat is a HeLa-derived cell line that constitutively produces HIV-1 Tat (26). Cells were treated with the indicated concentrations of hemin (the iron source), or desferrioxamine (the iron chelator), and compared to untreated cells. Total RNA was analyzed on Northern blots using a TfR-specific probe. In both cell lines, TfR mRNA levels can be regulated by iron; however, the extent of regulation is stronger in RD4 cells (Figure 2). Treatment of RD4

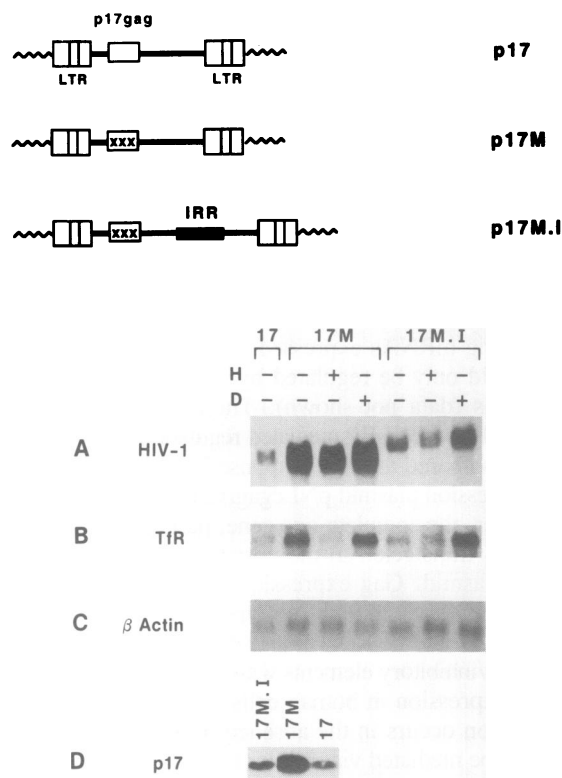
cells (Figure 2B) with 5 or 25  $\mu\text{M}$  hemin dramatically reduced the level of TfR mRNA. Even an overexposure of the Northern blot revealed only barely detectable levels of TfR mRNA in the presence of hemin. The presence of desferrioxamine increased TfR mRNA levels in RD4 cells at least 15-fold. In HLtat cells (Figure 2A), the presence of 10  $\mu\text{M}$  hemin had no significant effect, while 50  $\mu\text{M}$  hemin lowered TfR mRNA levels two-fold, indicating that these cells do not respond as strongly to the iron source as RD4 cells. Desferrioxamine treatment stimulated TfR mRNA accumulation only five-fold. Similar data were obtained for HLtat and the parent HeLa cells (not shown). Furthermore, IRR-containing mRNAs expressed from transiently transfected plasmids could only be regulated by iron in RD4 cells but not in HLtat cells (data not shown). Therefore, RD4 cells were chosen to study the IRE-BP mediated regulation of RNA stability.

We then compared the Rev response in RD4 and HLtat cells. The *gag* expression plasmid pNLc<sub>gag</sub>A2 that contains the HIV-1 LTR promoter, the complete *gag* gene, part of the *pol* gene, and the RRE was transfected in the absence or presence of a Rev expression plasmid. Gag expression was monitored by p24<sup>gag</sup> antigen capture assay. In both cell types, Gag expression is very poor in the absence of Rev. We had previously demonstrated that instability/inhibitory elements within *gag* gene are responsible for its low expression in human cells such as HeLa. Since this downregulation occurs in the absence of any viral factors, this is thought to be mediated via cellular factors (17). Similarly, low expression of Gag is detected in RD4 cells indicating that these downregulatory factors also exist in RD4 cells. While Rev activates Gag protein expression several hundred fold in HLtat cells, only about a 10-fold activation was observed in RD4 cells (data not shown). In conclusion, both cell lines allow the study of Rev function, although in RD4 cells Rev functions less efficiently.

### The presence of IRR downregulates accumulation of the stable mutant p17<sup>gag</sup> mRNA

It was previously demonstrated that the presence of IRR conferred instability to heterologous stable mRNAs (38, 39). In those studies, this region was inserted into the 3' untranslated regions of the HLA or human growth hormone genes and the DNAs were stably integrated into murine cells. Hybrid mRNAs containing IRR accumulated at lower levels, while the presence of the iron chelator desferrioxamine lead to a 10- to 30-fold up-regulation.

To establish whether this region also downregulated the stable mutant p17<sup>gag</sup> mRNA, the IRR was inserted into plasmid p17M resulting in p17M.I (Figure 1 and Figure 3 top panel). Plasmid p17M expresses a mutated form of the p17<sup>gag</sup> mRNA in which the HIV-1 instability element (INS-1) has been eliminated by site directed mutagenesis (16, 17). RD4 cells were transfected with the wild-type p17, the mutated p17M, and the hybrid p17M.I plasmids, and one day later total RNA and protein were extracted from duplicate plates. While high levels of *gag* mRNA were produced from p17M, the presence of IRR in p17M.I lowered the level of *gag* mRNA to that produced from the wild-type plasmid p17 (Figure 3, panel A). These data confirmed that the presence of a down-regulatory element within IRR also affected accumulation of the mutant p17<sup>gag</sup> hybrid mRNAs expressed from p17M.I. Rehybridization of the blot with a  $\beta$ -actin probe confirmed equal loading of the RNA samples (Figure 3, panel C). p17 and p17M.I also produced ten times less Gag protein compared to p17M (Figure 3, panel D). The insertion of IRR

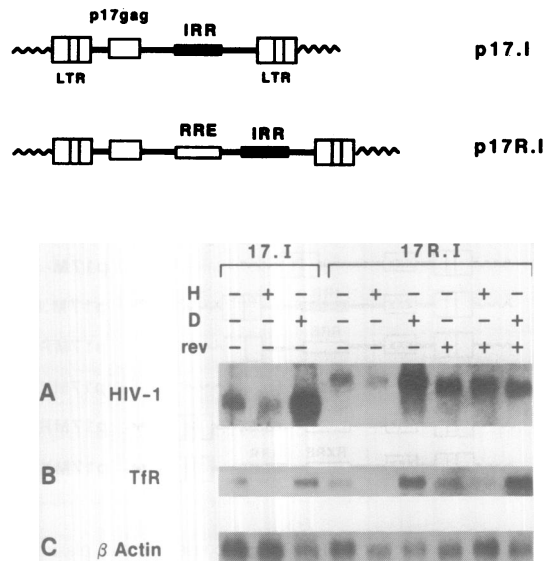


**Figure 3.** The presence of IRR downregulates accumulation of stable mutant p17<sup>gag</sup> mRNA. RD4 cells were transfected with p17, p17M, or p17M.I, together with the tat expression plasmid pL3tat, using the calcium phosphate coprecipitation technique. The structures of the plasmids are shown in the top panel (see also Figure 1). Two hours after transfection, the cells were washed and fresh medium was added with or without 10  $\mu$ M hemin (H) or 100  $\mu$ M desferrioxamine (D), as indicated at the top of the lanes. Next day, the cells were harvested and total RNA and protein were extracted. Duplicate Northern blots were hybridized to a probe spanning nt 8304–9008 of the molecular clone of HXB2 HIV-1 to detect the gag mRNAs (A) and to a 2.6-kb EcoRV–BamHI fragment of pTRS-1 (22) to detect the endogenous Tfr mRNA (B). The blots were rehybridized using a  $\beta$ -actin probe to detect actin mRNA (C). The p17<sup>gag</sup> protein produced in the absence of any treatment was visualized on a Western blot using an HIV-1 patient serum (panel D).

did not have any further effect on the already unstable *gag* mRNAs produced from p17 and p17R (data not shown).

#### Regulation of the IRR-containing hybrid mRNAs by IRE-BP

We next examined whether the IRR-containing hybrid mRNAs can be up-regulated in the presence of active IRE-BP. For this, RD4 cells transfected with p17M and p17M.I were incubated in medium with or without hemin or desferrioxamine. The next day, total RNA was extracted and analyzed on Northern blots. The presence of desferrioxamine (D) reproducibly increased the levels of the IRR-containing mRNA produced from the transfected plasmid p17M.I (Figure 3, panel A) and the endogenous Tfr mRNA (panel B) by three-fold and six-fold, respectively, compared to the levels of mRNA produced by the hemin (H) treated cultures. No iron regulation was observed for mRNAs that lack IRR, such as mRNAs from p17M (panel A), or p17, p17R, p17MR, a cotransfected luciferase expression plasmid (data not shown). These experiments indicate that the IRR confers instability to an otherwise stable viral mRNA (17M). These data also demonstrated that the hybrid IRR-containing

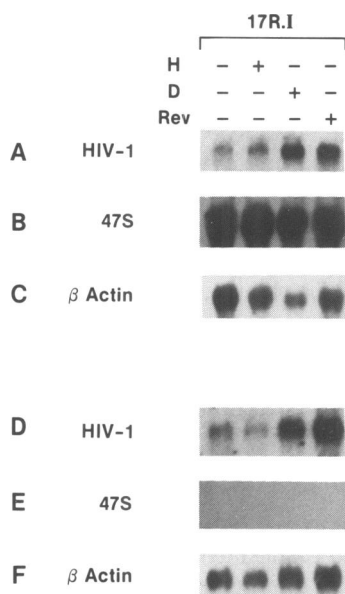


**Figure 4.** Regulation of IRR-containing mRNAs by IRE-BP and Rev. The structures of the plasmids are shown in the top panel (see also Figure 1). RD4 cells were transfected with the p17.I and p17R.I. HIV-1 Rev protein was provided by cotransfection of the Rev expression plasmid pCMV5rev as indicated at the top of the lanes. After transfection, the cells were treated overnight with 10  $\mu$ M hemin (H) or 100  $\mu$ M desferrioxamine (D) as indicated. Total RNA was isolated and analyzed on Northern blots by hybridization to the three probes indicated to the left.

mRNA (17M.I) can be appropriately regulated by the transferrin receptor regulatory elements.

#### Interaction of IRE-BP with IREs affects accumulation of mRNAs containing both the Tfr and the viral instability element INS-1

The viral mRNA encoded by the wild-type p17 plasmid has been shown to be unstable (17). To study regulation of this mRNA by IRE-BP, the IRR was inserted 3' to the p17<sup>gag</sup> gene in plasmids p17 and its RRE-containing analog p17R generating p17.I and p17R.I, respectively (Figure 1 and Figure 4 top panel). RD4 cells were transfected with the *gag*-IRR hybrid constructs p17.I and p17R.I. The cells were treated with hemin or desferrioxamine and total RNA was extracted the next day. The presence of hemin (H) did not significantly decrease the levels of IRR-containing *gag* mRNA produced from the transfected hybrid construct (Figure 4, panel A), while it lowered the levels of the endogenous Tfr mRNA (panel B). The presence of desferrioxamine (D) reproducibly increased the levels of the hybrid IRR-containing mRNAs produced from p17.I and p17R.I (panel A) as well as the endogenous Tfr gene (panel B). Quantitation of the blots revealed an approximate seven-fold increase of the hybrid IRR-containing mRNAs and at least 10-fold increase of the endogenous Tfr mRNA indicating a comparable increase in mRNA accumulation. To control for equal loading, the blot was rehybridized with the  $\beta$ -actin probe (panel C). These IRR-containing mRNAs responded to iron regulation both in the presence (17.I) and absence (17R.I) of the RRE element. Since the presence of the viral instability/inhibitory element INS-1 (in p17.I, p17R.I) did not impair regulation by the IRE-BP, we conclude that the hybrid mRNAs are accessible to iron regulation.



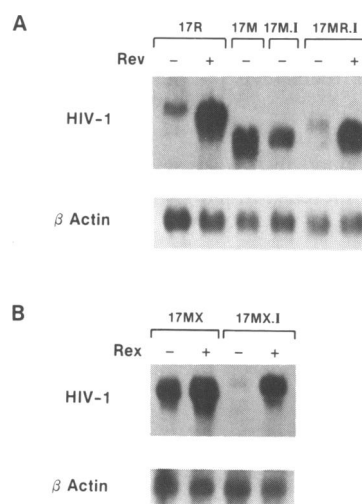
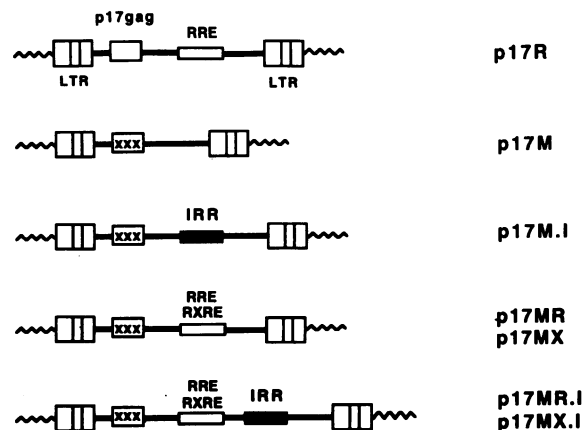
**Figure 5.** Subcellular localization of hybrid p17R.I mRNAs. Duplicate plates of RD4 cells were transfected with p17R.I in the presence or absence of the HIV-1 rev expression plasmid pCMVsrev, hemin (H), or desferrioxamine (D), as indicated. One day later, total (panels A, B, C) and cytoplasmic mRNAs (panels D, E, F) were isolated and analyzed on Northern blots. The blots were hybridized with the different probes indicated to the left. The gag mRNA was visualized after hybridization to the HIV-1 probe (panels A, D). To exclude contamination of the cytoplasmic fraction with nuclear RNA the blots were rehybridized with a probe detecting the 47S ribosomal precursor mRNA as described previously (34) (panels B, E). To control for loading, the blots were rehybridized to the  $\beta$ -actin probe (panels C, F). The structures of the plasmids are shown in Figure 1.

These data demonstrate that the positive interaction of the IRE-BP with the IREs is dominant over the negative effect of the viral instability element.

**Regulation of RRE-containing hybrid mRNAs by Rev in RD4 cells**

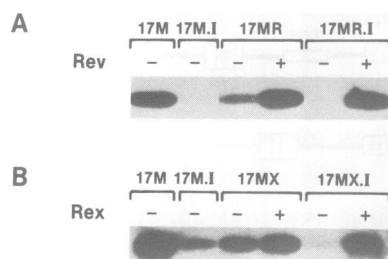
We further tested whether the presence of IRR on the hybrid mRNAs affected Rev regulation in RD4 cells by cotransfection with a Rev-producing plasmid. The presence of Rev increased the mRNA levels from p17R.I (Figure 4, panel A) approximately four-fold. A similar increase in the presence of Rev has been observed for other RRE-containing constructs such as p17R (Figure 6, panel A). These results demonstrate that the Rev-RRE interaction can overcome the INS-1 effect irrespective of the presence of the cellular instability element located within IRR. These experiments suggest that Rev interacts with these mRNAs and positively affects their expression. It is interesting to note that desferrioxamine treatment in the presence of Rev did not further affect the levels of mRNA that contain both the RRE and IRR in cis (p17R.I, Figure 4, panel A).

In order to determine the localization of the hybrid mRNAs in the presence of Rev or active IRE-BP, duplicate plates of RD4 cells were transfected with p17R.I as described above. One day later, total and cytoplasmic mRNAs were prepared. Northern blots of total mRNA confirmed the previously observed increase of the 17R.I mRNAs by both Rev and desferrioxamine treatment (Figure 5, panel A, see also Figure 4). This increase is paralleled by an increase in the cytoplasmic mRNA levels (Figure 5, panel



**Figure 6.** The Rev-RRE or Rex-RXRE interaction affects the accumulation of hybrid IRR-containing mRNAs. The structures of the plasmids are shown in the top panel (see also Figure 1). HLtat cells were transfected with the indicated constructs in the presence or absence of a Rev expression plasmid pLsrev (panel A) or Rex expression plasmid pRSVrex (panel B), as indicated. Total mRNA was analyzed on Northern blots with probes indicated to the left. Luciferase activity was determined to control for transfection efficiency. The luciferase values are (from left to right) for panel A: 628, 284, 179, 260, 273, and 156 respectively; for panel B: 380, 451, 214, and 223, respectively.

D). The blots were rehybridized with a probe specific for ribosomal precursor RNA (47S rRNA) to control for purity of cytoplasmic preparation (panels B and E) as well as with a probe for  $\beta$ -actin to control for equal loading of the RNA samples (panels C and F). It is interesting to note that IRE-BP is localized in the cytoplasm and the interaction with the IRR-containing mRNAs is thought to occur predominantly in the cytoplasm (21). Rev accumulates in the nucleolus and its function has been proposed to include both nuclear and cytoplasmic events. Our data demonstrate that the presence of Rev or active IRE-BP results in elevated levels of hybrid IRR/RRE-containing mRNAs in the cytoplasm. We hypothesize that Rev recognizes these hybrid mRNAs in the nucleus and affects their fate. On the other hand, IRE-BP could bind to the cytoplasmic hybrid mRNAs and affect its accumulation.



**Figure 7.** The Rev-RRE or Rex-RXRE rescues interaction increases p17<sup>gag</sup> protein expression from IRR-containing mRNAs. HLtat cells were transfected with the indicated plasmids (for details see Figure 1). The p17<sup>gag</sup> protein was visualized on Western blot using an HIV-1 patient serum. Luciferase activity was determined to control for transfection efficiency. The luciferase values are (from left to right) for panel A: 199, 130, 172, 112, 185, and 119 respectively; for panel B: 348, 371, 265, 139, 274, and 185, respectively.

Although the effects of Rev and IRE-BP at the RNA levels were similar, their effects on p17<sup>gag</sup> protein production were different. Western blots to detect p17<sup>gag</sup> showed an increase in Gag protein produced in the presence of Rev (data not shown) that parallels the increase in p17<sup>gag</sup> mRNA. We failed to detect significant effects of active IRE-BP on p17<sup>gag</sup> protein production from p17R.I. The most likely explanation is that while the IRE-BP/IREs interaction counteracts INS-1-mediated mRNA instability, it is less efficient in counteracting a putative translational defect conferred by INS-1 in RD4 cells. Therefore, Rev and IRE-BP act on the RNA via distinct mechanisms, and they are not fully interchangeable. Further experiments are required to analyze the different mechanisms in detail, and to determine the effects of the different factors in stable expressing cell lines.

#### Downregulation conferred by IRR can be overcome by the Rev-RRE and Rex-RXRE interaction

We explored whether in human HeLa cells the down-regulatory effect of the cellular instability element within IRR can be counteracted by the interaction of Rev with the RRE element or by the interaction of Rex of HTLV-I with the RXRE element. Therefore, the RRE of HIV-1 and the RXRE of HTLV-I were inserted into p17M.I resulting in p17MR.I and p17MX.I, respectively (Figure 1 and Figure 6 top panel). These plasmids contain the mutant p17<sup>gag</sup> gene that lacks the viral instability element INS-1. In addition, they contain IRR as instability element and RRE or RXRE as binding site for the positive viral regulator. The HeLa-derived cell line HLtat was transfected with the different constructs in the absence and presence of the Rev- or Rex-expressing plasmids and the production of mRNA (Figure 6A and 6B) and p17<sup>gag</sup> protein (Figure 7A and B) was analyzed.

Upon transfection into HLtat cells, we observed that the presence of IRR in the mRNA produced from plasmid p17M.I lowered gag expression when compared to p17M (Figures 6A, 7A). Similar results were obtained in RD4 cells (see Figure 3). The insertion of the HIV-1 RRE lowered expression of p17MR.I (Figure 6A, 7A) and p17MR (Figure 7A), resulting in barely detectable levels of p17<sup>gag</sup> mRNA and protein. These results are consistent with additional data from our laboratory indicating that the RRE region in the absence of Rev has a negative effect on the RNA accumulation (20). These results further indicated that the 17MR.I mRNA carries both a strong cellular instability

element (located within IRR) and a viral RNA instability element (located within RRE). The presence of Rev increased mRNA and protein expression of p17MR.I and p17MR to levels comparable to that of p17M. Similarly, the presence of Rev increased the levels of mRNA produced from p17R, a plasmid that contains the wild-type p17<sup>gag</sup> gene linked to RRE (Figure 7A). To control for transfection efficiency, a luciferase expression plasmid was cotransfected and the values of the measured luciferase activities are shown in the figure legend. The luciferase values indicate that the observed differences in gag mRNA levels are not due to unequal transfection efficiencies but rather due to the difference in produced mRNAs. The slight drop in luciferase activity in the presence of Rev is consistent with our previous observation (24). In summary, these data demonstrate that the interaction of Rev with the RRE element can rescue an mRNA containing in addition a strong heterologous instability element.

To determine the extent of the crossactivation among different elements, we also studied constructs containing the RXRE element of HTLV-I. Insertion of this element in p17M resulted in plasmid p17MX (Figure 1 and Figure 6 top panel). HLtat cells were transfected with this plasmid in the absence or presence of a Rex-expressing plasmid. One day later, the cells were harvested and analyzed for mRNA (Figure 6B) and protein (Figure 7B) production. The presence of RXRE element lowered Gag expression by about 3-fold when compared to p17M, while the Rex protein did not significantly increase expression from p17MX (1.5-fold increase). These data indicate that, in contrast to the HIV-1 RRE, the HTLV-I RXRE does not contain downregulatory elements that render the hybrid mRNA Rex-dependent. We then inserted the IRR into p17MX, resulting in p17MX.I (Figure 1 and Figure 6 top panel). The presence of the IRR as the only instability determinant lowered Gag expression from the hybrid mRNA drastically, while the presence of Rex increased Gag expression to about the level produced by p17MX. Analyses of expression from p17MX in the absence and presence of Rex indicate that the accumulation of the hybrid mRNAs parallels p17<sup>gag</sup> protein expression (compare Figures 6B and 7B). A luciferase expression plasmid was cotransfected to control for transfection efficiency and the values are shown in the figure legend. These results demonstrate that the effect of the cellular instability element within IRR could be counteracted by the interaction of either Rev or Rex regulatory viral proteins with their corresponding RNA elements.

## DISCUSSION

In this report, we demonstrate that low mRNA accumulation due to posttranscriptional blocks caused by the presence of the instability/inhibitory element INS-1 of HIV-1 can be elevated by the posttranscriptional regulatory system of the Tfr mRNA. The IRE-BP had been shown to stabilize the Tfr mRNA via direct binding to the IREs (22), which is thought to prevent association of putative cellular destabilizing factors with the instability determinants present within this region. The hybrid mRNAs used in this study contain the viral INS-1 element as an additional strong instability determinant located about 400 nt 5' of the IRR. Since these hybrid mRNAs are regulated by active IRE-BP to a similar extent as the Tfr mRNA, we conclude that the Tfr stabilization mechanism is not limited to the inhibition of binding of putative downregulatory factors to the Tfr mRNA instability



determinant. The mechanism that permits the rescue of an RNA containing an instability element distant from the site of IRE-BP/IREs interaction remains to be elucidated. Since the hybrid mRNAs used in this study did not contain any functional splice sites, the presence of distinct elements (such as IRR, INS-1) were responsible for the low level of accumulation in the absence of active IRE-BP or Rev/Rex factors. The IRE-BP/IREs interaction only partly relieved the inhibitory effect of the INS-1, as the increase in the mRNA accumulation was not followed by an increase in p17<sup>gag</sup> protein synthesis. To explain these results, we hypothesize that, although IRE-BP exhibits Rev-like mRNA stabilization activity, it cannot substitute for Rev to counteract a translational defect caused by INS-1 in RD4 cells.

We also demonstrate that the expression from hybrid mRNAs that contain the IRR as the only strong instability determinant and the positive RNA elements (RRE or RXRE) can be rescued by Rev or Rex, respectively. This is the first demonstration that viral mRNA rescue mechanisms can counteract the low expression caused by a cellular instability element. These data suggest that the underlying mechanisms causing the 'defect' in mRNA expression may be interchangeable. The Rev and Rex factors of the different HIV/HTLV viruses have been shown to be interchangeable in many but not all cases (for recent review see (8)). Function of these factors requires their efficient binding to the specific RNA target elements. As a result, the Rev/Rex factors of various viruses counteract the effects of the heterologous viral inhibitory/instability elements. We have identified several inhibitory/instability elements in HIV-1 (16, 20) (R. Schneider and G.N. Pavlakis, unpublished results), as well as in HTLV-I and SIV (L. Solomin, M. Saltarelli, B.K. Felber, G.N. Pavlakis, unpublished results). These elements are distinct in nucleotide sequence and can act when present in heterologous mRNA. Since Rev and Rex systems can overcome heterologous viral and cellular (as shown here on the example of IRR of the TfR mRNA) posttranscriptional downregulatory elements, they may represent mRNA rescue systems with general applications permitting efficient expression of a variety of heterologous 'defective' or unstable mRNAs. Experiments are currently ongoing to test this hypothesis. This model is further corroborated by the fact that the Rev/Rex factors are involved in several posttranscriptional steps. Rev, the best studied factor, has been shown to affect the half-life, transport from the nucleus to cytoplasm, and the translation of RRE-containing viral mRNAs (16, 28, 34, 40, 41, 42, 43, 44, 45, 46). Rev is thought to chaperone RRE-containing mRNAs through the entire pathway from the nucleus to the cytoplasm which is supported by recent findings of Rev shuttling (12, 13).

The data presented here, in agreement with other findings (20, 47), indicate that RRE contains a downregulatory element detectable in human cells in the absence of Rev. This is reminiscent of IRR, which is also a negative determinant for mRNA expression in the absence of IRE-BP. In contrast, the HTLV-I RXRE does not contain downregulatory elements.

Taken together, these and previously published data suggest that cellular destabilization mechanisms can act on viral and cellular mRNAs. On the other hand, specific rescue mechanisms such as Rev/RRE, Rex/RXRE or IRE-BP/IREs can counteract different negative determinants of viral or cellular origin. It is unclear whether a common underlying mechanism is responsible for this downregulation. The identification of the cellular factors that cause the viral and cellular mRNA defects will allow a better understanding of the regulation of mRNA utilization.

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