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Cellular Protein Modulates Effects of Human Immunodeficiency Virus Type 1 Rev

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Replication of human immunodeficiency virus type 1 requires expression of the viral *trans* activator Rev. Rev binds to a highly structured RNA, the Rev response element, which is present in singly spliced and unspliced genomic viral RNAs. Although Rev helps to transport these transcripts from the nucleus to the cytoplasm, the mechanism(s) involved is not fully understood. Using the yeast two-hybrid system, we isolated a murine protein (YL2) that interacts with the basic domain of Rev, which is essential for the function of Rev in vivo and for the inhibitory splicing activity of Rev in vitro. YL2 has 92% identity to a human 32-kDa protein (p32), which copurifies with alternative splicing factor SF2/ASF. Furthermore, we found that whereas expression of YL2 greatly potentiated the activity of Rev, antisense YL2 transcripts blocked the effects of Rev in mammalian cells. YL2 also increased the activities of Rex on the Rex response element and of hybrid Rev proteins fused to Tat and the coat protein of bacteriophage MS2 on their respective RNAs. Thus, YL2 or p32 is a cellular protein that modulates the function of human immunodeficiency virus type 1 Rev.

Rev is one of the earliest proteins expressed by human immunodeficiency virus type 1 (HIV-1) and is involved in the posttranscriptional regulation of viral RNAs (6, 11, 27, 28, 46). It is a 19-kDa nuclear protein that contains four functional domains (38). Of these, the basic domain binds to the Rev response element (RRE) (22, 30, 31, 39, 49, 52), which forms highly structured RNA stem-loops (41). Since this basic domain also inhibits RNA splicing in vitro (32, 33) and a basic sequence is required for the optimal function of Rev even when it is tethered to RNA via a heterologous RNA-binding protein such as the coat protein of bacteriophage MS2 (45, 50), the basic region of Rev performs functions in addition to RNA binding. Furthermore, the two regions flanking this basic domain are required for multimerization of Rev on the RRE (38, 42). Finally, five leucines near the C terminus form the activation domain of this protein (38).

Splicing and transport of primary HIV-1 transcripts are complex. In the absence of Rev, only multiply spliced viral transcripts that direct the synthesis of Tat, Rev, and Nef are exported from the nucleus (29, 48). These proteins perform important regulatory functions but are not themselves packaged into virions. In the presence of Rev, singly spliced and unspliced genomic viral transcripts also appear in the cytoplasm (7, 10, 19, 29). Whereas singly spliced RNA codes for envelope glycoproteins gp120 and gp41, the unspliced RNA codes for Gag proteins, integrase, reverse transcriptase, and protease, which together with this RNA are packaged into the budding virion. Thus, Rev plays an essential role in the life cycle of HIV-1 (7).

However, the mechanism of *trans* activation by Rev is not well understood. Since singly spliced and unspliced viral transcripts are detected in the nucleus but not in the cytoplasm in the absence of Rev, Rev might itself transport viral RNAs which contain the RRE (7, 10, 19). Alternatively, since the basic domain of Rev also inhibits RNA splicing in vitro, Rev could affect RNA splicing and its effects on RNA transport would be secondary (32, 33). Indeed, the latter mechanism has been observed with artificial substrates, where Rev rescued incompletely spliced but not spliced transcripts or transcripts containing no splicing signals via the RRE in the *env* intron (20, 36) or introduced into the β-globin intron (3, 4). Of course, these posttranscriptional mechanisms are not mutually exclusive and either could explain the effects of Rev.

To better understand the function of Rev, we isolated cellular proteins that interact with Rev by using the yeast two-hybrid system (5, 12, 53). One cDNA encoding a protein that interacts with the basic domain of Rev was characterized and expressed. This protein, which we call YL2, had no effect on its own but strongly influenced the activity of Rev on the RRE in primate cells. Since YL2 is most likely the murine homolog of p32 (35), an acidic protein that copurifies with alternative splicing factors SF2/ASF (17, 35), our finding supports a role for Rev in the early splicing events of HIV-1 transcripts.

MATERIALS AND METHODS

Plasmids. Yeast plasmid pMA424, which contains the Gal4 DNA-binding domain (amino acids [aa] 1 to 147), was obtained from M. Ptashne (2). Rev was cloned into its *Eco*RI-*Sal*I sites (pMARev) (26). pMARevM10 contains a mutation (LE to DL at aa 78 and 79) in the activation domain of Rev (38). In pMARevΔR, the basic domain (aa 34 to 50) of Rev was deleted. In pMARevM6, the RRRR sequence (aa 42 to 45) in the basic domain of Rev was replaced by DL (38). The mouse embryonic cDNA library was obtained from D. Nathans (5). pDM121, which contains the Rev cDNA under the control of the Rous sarcoma virus promoter, and pCMV128, which contains chloramphenicol acetyltransferase (CAT) and RRE sequences in the middle of the *env* gene, was as previously described except that the promoter was changed from the simian virus 40 promoter to the cytomegalovirus promoter (26). All effector and target plasmids were the kind of gift of T. Parslow.

pCMV (pcDNA/Amp), which has a cytomegalovirus pro-

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moter followed by multiple cloning sites, was purchased from Invitrogen (San Diego, Calif.). This plasmid was used as the control for all of our experiments involving the cytomegalovirus promoter. pYL2 is the mouse cDNA that was cloned in *Saccharomyces cerevisiae*. YL2 cDNA was then subcloned into pCMV with a consensus ATG translational initiation sequence. This construction is called pCMVYL2. YL2 cDNA was also cloned into pCMV in the anti-sense orientation to create the construction called pCMVαYL2.

p138PROBE was constructed by removing the *Bam*HI-*Eco*RV fragment, which spans the 3′ splice site of the HIV-1 *env* intron, from pCMV138 (23) and ligating it into the pBluescript KS *Bam*HI-*Eco*RV sites. This construction was used to synthesize RNA probes for RNase protection assays.

Yeast transformation and filter color assay. *S. cerevisiae* PCY2 was obtained from D. Nathans (5). This yeast strain contains the integrated *lacZ* gene under control of the Gal1 promoter. Yeast transformation was done by using a polyethylene glycol-cation transformation kit purchased from Bio 101 (La Jolla, Calif.). All yeast media were ordered from Bio 101.

For screening of the cDNA library, PCY2 was first transformed with pMARev with the LEU2 yeast selectable marker. The cDNA library with the TRP1 selectable marker was then transformed into *S. cerevisiae* which already contained pMARev. This two-step transformation protocol resulted in higher cotransformation efficiencies than the one-step cotransformation technique. After the cDNA was cloned, all other transformations were done by one-step cotransformation with a DNA mixture containing 5 μg of each plasmid. In brief, transformed yeasts were directly plated on filters with the selection medium (dextrose lacking leucine and tryptophan). After colonies grew, the filters were lifted, immersed in liquid nitrogen for 5 s, and dried in air. The filters were then placed on plates containing 2 ml of Z buffer and 4% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The filters were incubated in a 37°C incubator (without CO_2) until the colonies turned blue. Colonies were recovered and expanded. Yeast plasmids were extracted by a DNA preparation kit from Bio 101. In other experiments, yeasts were plated on the selection medium without filters and transferred to filters when the colonies grew. The filter color assay was the same. Quantitation was done as previously described (5, 12, 53).

Mammalian cell transfections and CAT assay. CV1 cells were used in all of our transfection assays. The $CaPO_4$ precipitation transfection protocol was used (37). In brief, cells were grown to one-third confluency, and fresh medium was added 2 to 4 h before transfection. Ca^{2+}-precipitated DNA was added dropwise to the medium and incubated overnight. The medium was changed 16 to 20 h after transfection, and the cells were grown for an additional 24 to 30 h and then harvested. A 1-μg sample of CAT reporter plasmid pCMV128, pDM138XRE, pDM12TAR, or pDM257 was used in all transfections (23, 25, 26, 37). Total DNA was held constant at 11 μg and was balanced by addition of the pCMV vector. Variable amounts of plasmids coding for Rev (pDM121), Rex (pDM121REX), Tat/Rev (pcTat/Rev), Rev-MS2 (pDM191), and YL2 (pCMVYL2 and pCMVαYL2) were used (23, 25, 26, 37). CAT assays were done as previously described (37). Protein concentrations were used to normalize minor differences between transfections.

RNase protection assay. Transfected cells were scraped off of the plates, and a small portion (1/20) of the pellet was used for CAT assays. Cells were spun down and washed with cold phosphate-buffered saline. They were then lysed in Nonidet P-40 lysis buffer for 10 min on ice. After the cells were spun again, supernatants containing cytoplasmic RNA were harvested and layered on a CsCl cushion. Nuclear pellets were washed with Nonidet P-40 lysis buffer and dissolved in GnHCl to collect nuclear RNA. RNA samples were spun overnight in an SW41 rotor at 30,000 × *g* to separate RNA from DNA. A round, clear pellet was visible and was dissolved in water for 30 min. DNase I was also used to remove any residual DNA.

p138PROBE was transcribed with T3 RNA polymerase to produce [α-^{32}P]UTP- or [α-^{32}P]GTP-labelled RNA probes. p138PROBE was linearized with *Hin*fI, labelled, and then used as recommended by the supplier (Clontech, Palo Alto, Calif.). RNase protection products were resolved on a 7 M urea–4% polyacrylamide gel and exposed to X-ray film.

RESULTS

Identification of a cellular protein that interacts with Rev in the yeast two-hybrid system. The yeast two-hybrid system was used to identify cellular proteins that interact with Rev (5, 12, 53). The strategy is shown in Fig. 1A. Whereas Rev was fused to the N-terminal DNA-binding domain (aa 1 to 147) of Gal4, cellular proteins were fused to the C-terminal activation domain (aa 768 to 881) of Gal4 (12). For our experiments, we employed PCY2, an *S. cerevisiae* strain that contains the *lacZ* gene under the control of the Gal1 promoter and six Gal4 DNA-binding sites (5). PCY2, which was first transformed with pMARev encoding the hybrid Rev-Gal4 protein, was subsequently transformed with a mouse embryonic fusion cDNA library. Colonies were assayed for β-galactosidase activity in situ. Colonies that turned blue indicated positive interactions between Rev and the cellular protein.

In the first screening of 10^5 colonies, 1 (pYL2) became blue within 3 h. This colony was recovered from the filter and amplified. To examine the specificity of its interaction with Rev, the isolated plasmid was cotransformed with or without Rev (pMARev) into PCY2 (Fig. 1B, YL2 + MARev and YL2). After deletion of 196 aa (from aa 12 to 208; *Eco*RI site), YL2 no longer interacted with Rev (Fig. 1B, YL2R1 + MARev). YL2 in combination with just the DNA-binding domain of Gal4 also gave no blue colonies (Fig. 1B, YL2 + MA242). Thus, Rev interacts specifically with YL2 in *S. cerevisiae*.

Since deletion of the entire basic domain (pMARevΔB, aa 34 to 50) and mutations of the central four arginines to aspartic acid and leucine (pMARevM6, aa 41 to 44), but not of the activation domain of Rev (pMARevM10, aa 78 and 79) (38), completely abolished interactions between Rev and YL2, YL2 interacts with the basic domain of Rev (Fig. 2). Other controls consisted of only the activation domain of Gal4 (aa 768 to 881), transcription factor c-Fos, and YL1, which is a protein that interacts with the activation domain of Tat in the yeast two-hybrid system (Fig. 2). Since none of these proteins interacted with Rev, we conclude that YL2 specifically interacts with the basic domain of Rev in *S. cerevisiae*.

Dideoxy sequencing of the entire YL2 cDNA revealed that the amino acid sequence of YL2 has strong homology (>92% identity) to the 32-kDa protein (p32) which copurifies with human alternative splicing factor SF2/ASF (Fig. 3) (35). Interestingly, YL2, like p32, contains a leucine initiator codon (Fig. 3) (35). For this reason and since amino acid substitutions between murine YL2 and human p32 are conservative, it is very likely that YL2 is the mouse p32.

YL2 can significantly increase Rev activity in mammalian cells. To prove that YL2 is the cellular factor which interacts with Rev, we subcloned YL2 into a eukaryotic expression vector (pCMVYL2) and cotransfected PCMVYL2 with or without Rev (pDM121) and a Rev-responsive target plasmid

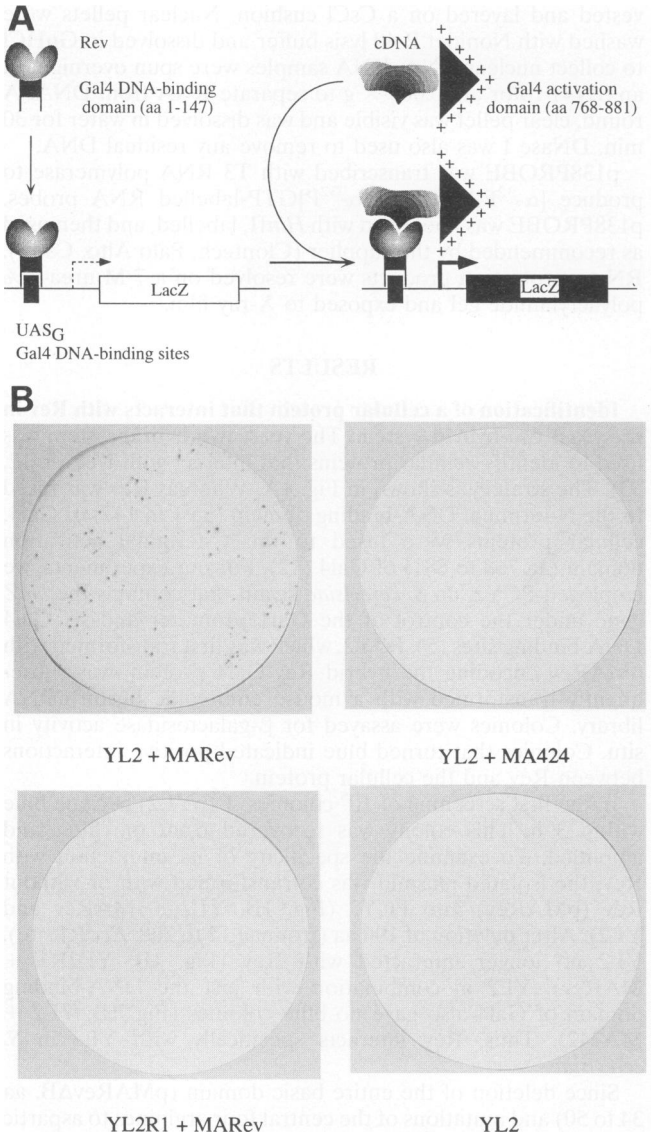
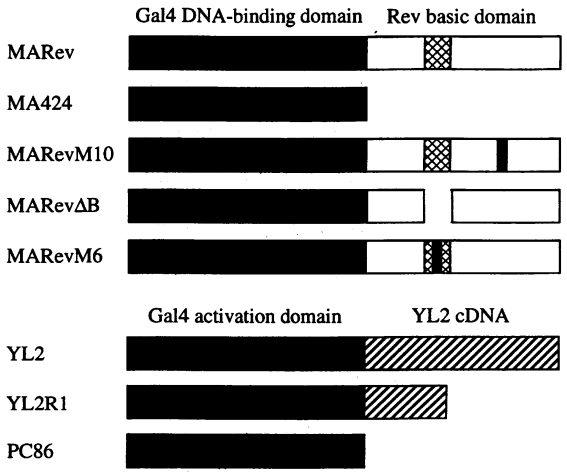


FIG. 1. Detection of the Rev-binding protein by the yeast two-hybrid system. (A) Design of the yeast two-hybrid system. Rev was fused to the C-terminal DNA-binding domain (aa 1 to 147) of Gal4 (pMARev). The cDNA library was linked to the C-terminal activation domain (aa 768 to 881) of Gal4. The *lacZ* reporter gene, which was under the control of six DNA-binding sites for Gal4 placed upstream of the Gal1 promoter, was expressed stably in yeast strain PCY2. Productive interactions between Rev and the cellular protein resulted in blue colonies. (B) Typical filter color assay. Presented are cotransfections with YL2 and Rev (YL2 + MARev), YL2 and the DNA-binding domain of Gal4 alone (YL2 + MA424), deletion of aa 12 to 208 of YL2 and Rev (YLR1 + MARev), and YL2 alone (YL2).



CONSTRUCTIONS	COLOR	QUANTITATION
MARev	White	ND
YL2	White	2.0
MARev + YL2	Blue	29.0
MARev + YL2R1	White	2.5
MARev + YL1	White	ND
MARev + PC86	White	ND
MARevM10 + YL2	Blue	30.0
MARevΔB + YL2	White	2.8
MARevM6 + YL2	White	5.4
MARev + Fos	White	ND

FIG. 2. Mapping of interactive domains in Rev and YL2. The results of the filter color assay are summarized below the diagrams of plasmid constructions. The Gal4 DNA-binding domain was fused to wild-type Rev (MARev), Rev with a deletion of the basic domain (MARevΔB), and Rev with mutations in the activation and basic domains (MARevM10 and MARevM6). YL2 and truncated YL2 (YL2R1) were linked to the activation domain of Gal4. MA424 and PC86 were parental plasmids coding for the DNA-binding and activation domains of Gal4, respectively. Color intensities of yeast colonies were quantitated as described in Materials and Methods, and the results are presented at the bottom. ND, not done.

(pCMV128) into CV1 cells (26). Since pCMV128 contains both the CAT gene and the RRE in the middle of the *env* intron, levels of *trans* activation were measured by CAT assays (Fig. 4A and 5A) (26). With pCMV128, high levels of CAT activity are observed only if unspliced viral RNA is transported out of the nucleus by Rev (26). As shown in Fig. 4A, YL2 increased the effects of Rev 31-fold in these cells. This number was obtained by dividing the effects of Rev and YL2 (493-fold) by those of Rev alone (15.8-fold) and pCMV128 (Fig. 4A). Moreover, cotransfection with YL2 resulted in only fourfold-increased CAT activities in the absence of Rev (Fig. 4A). Thus, high levels of *trans* activation of pCMV128 require coexpression of Rev and YL2 and YL2 has little to no activity on the RRE without Rev.

To rule out the possibility that YL2 had nonspecifically increased transcription and/or translation of Rev in our CAT assays, pCMVYL2 and pRSVCAT were cotransfected into CV1 cells. Antisense YL2 (pCMVαYL2) was used as the negative control. pRSVCAT was chosen as the target plasmid because Rev in pDM121 is transcribed from the Rous sarcoma virus promoter. As pDM121 contains no intron, YL2 could not affect the processing of Rev RNA. Since no change in CAT activities was observed (Fig. 4B), our results demonstrate that YL2 has no effect on the Rous sarcoma virus promoter or the CAT RNA and protein. Thus, the synergistic effects of YL2 and Rev cannot be explained by Rev-independent increases in gene expression.

YL2 increases the accumulation of unspliced viral cytoplasmic RNA. To demonstrate that increased CAT activities were also reflected at the RNA level, we performed RNase protection assays and measured ratios of unspliced RNAs in the cytoplasm and nuclei of transfected cells. An RNA probe spanning the 3′ splice acceptor site was used to distinguish

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          1        10        20        30        40        50
YL2   LHTEGDKDFVEFLTDEIKEEKKIQKHKSLPKMSGDWELEVNGTEAKLLRK
      |||:|||:||:||:|||||||:|||||||:||||:||||||||:||
P32   LHTDGDKAFVDFLSDEIKEERKIQKHKTLPKMSGGWELELNGTEAKLVRK

          51        60        70        80        90       100
      VAGEKITVTFNINNSIPPTFDGEEEPSQGQKAEEQQPELTSSPNFVGDVT
      |||||||||||||||||||||||||||||:|||:|||||:||||::|:
      VAGEKITVTFNINNSIPPTFDGEEEPSQGQKVEEQEPELTSTPNFVVEVI

         101       110       120       130       140       150
      K-TDGKKTLVLDCHYPEDEIGHEDEAESDIFSIKEVSFQATGDSEWRDTRY
      |::||||:|||||||||||:|:|||||||||:|||||:||:|||:||:|
      KNDDGKKALVLDCHYPEDEVGQEDEAESDIFSIREVSFQSTGESEWKDTNY

         151       160       170       180       190       200
      TLNTDSLDWALYDHLMDFLADRGVDNTFADELVELSTALEHQEYITFLEDL
      ||||||||||||||||||||||||||||||||||||||||||||||||||
      TLNTDSLDWALYDHLMDFLADRGVDNTFADELVELSTALEHQEYITFLEDL

             208
      KSFVKNQ
      |||||:|
      KSFVKSQ
```

FIG. 3. Amino acid sequence of YL2. The sequences of the YL2 open reading frame and the human 32-kDa protein (p32), which is associated with alternative splicing factor SF2/ASF, are compared. Vertical bars denote identical amino acids, and colons represent mismatches. The sequences are 92% identical. Note that YL2 and p32 have a CTG initiation codon, which codes for leucine.

between spliced and unspliced transcripts (Fig. 5A). Our results are presented in Fig. 5B. Compared with cells cotransfected with the target plasmid (pCMV128) and Rev (Fig. 5B, left panel, lane 3), fivefold-higher levels of unspliced RNA (indicated by the arrow) were detected in the cytoplasm of cells cotransfected with pCMV128, Rev, and YL2 (Fig. 5B, left panel, lane 5). This stimulation was less than that observed with CAT assays (32-fold versus 5-fold). This was also true of the effects of Rev on pCMV128, with which levels of CAT activity increased 16-fold and those of the RNA increased less than 5-fold (compare Fig. 4A and 5B, left panel, lanes 2 and 3). These differences in RNA levels and protein activity had been noted previously and are probably due to the rapid degradation of unspliced viral RNAs in the nucleus (40) and the great stability of the CAT protein in the cytoplasm (18). It is of note that no changes were observed in the total amounts of spliced transcripts with Rev, YL2, or both. Importantly, there was also no significant change in the levels of unspliced RNA in nuclei (Fig. 5B, right panel, lanes 2 to 5). These invariant spliced (Fig. 5B, left panel, lanes 2 to 5) and nuclear (Fig. 5B, right panel, lanes 2 to 5) RNA species also served as controls for transfection efficiencies and amounts of RNA in our RNase protection assays. Thus, YL2 does not simply increase the total levels of the unspliced RNA substrate for Rev; rather, it increases the transport of unspliced viral RNA from the nucleus to the cytoplasm in the presence of Rev and the RRE.

Blocking of endogenous YL2 expression reduces the activity of Rev in cells. To investigate whether YL2 is also required for the Rev function in cells, we used antisense YL2 to reduce cellular levels of YL2. pCMVαYL2, which expresses YL2 cDNA in the antisense orientation, was cotransfected with Rev and pCMV128 into CV1 cells. Results of these cotransfections are presented in Fig. 6. A steady decline in the activity of Rev was observed in the presence of increasing amounts of antisense YL2 (pCMVαYL2). The decline began at the lowest concentration of pCMVαYL2 (0.5 μg) and was fourfold by 4 μg of pCMVαYL2, which is only twofold greater than the amount of pDM121 (2 μg). No similar decreases were observed with antisense Tat RNA and pCMV128 and with antisense YL2 and pRSVCAT (data not presented). Since low concentrations of antisense YL2 could effectively inhibit the Rev function in cells, we conclude that YL2 is an important cellular cofactor for Rev.

YL2 also potentiates the activities of Rex on the XRE and those of hybrid Tat-Rev and Rev-MS2 proteins on their RNA targets. Since Rev of HIV-1 and Rex of human T-cell leukemia virus type I function similarly via their respective RNA targets, which are the RRE and the Rex response element (XRE) (1, 9, 21), and since hybrid Rev-Rex proteins can function via either the RRE or the XRE (23), it was important to determine that YL2 also potentiates the activity of Rex via the XRE. Similar cotransfections were performed with Rex and YL2 effectors and the XRE targets (Fig. 7), as previously described for Rev on the RRE (Fig. 4) (23). Indeed, YL2 increased the activity of Rex fivefold (Fig. 7). Finally, since a basic sequence is still required for optimal Rev activity via heterologous RNA-tethering mechanisms, we examined whether YL2 could potentiate activities of hybrid Tat-Rev and Tat-MS2 proteins

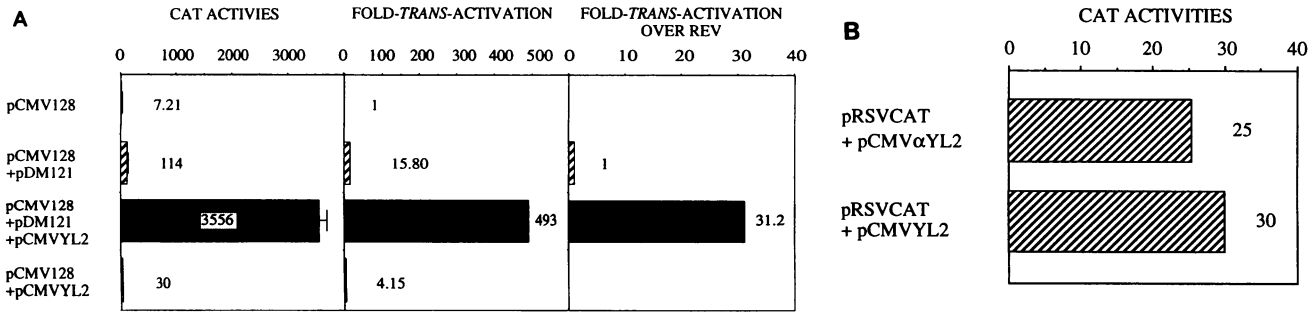

FIG. 4. YL2 increases Rev activity in CV1 cells. (A) Cotransfections of RRE target plasmid pCMV128 (1 μg) with the effector plasmid(s) coding for Rev (pDM121; 2 μg) and/or YL2 (pCMVYL2; 9 μg) into CV1 cells. CAT activities, fold *trans* activation, and fold *trans* activation over that of Rev for informative transfections are presented. Cotransfections with single effectors are represented by the striped bars, and those with both effectors are represented by the gray bars. Fold *trans* activation represents the level of CAT activity above that obtained with the target plasmid (pCMV128) alone. Fold *trans* activation over that of Rev represents the level of CAT activity obtained with both effectors over that obtained with Rev alone. Experiments were performed in triplicate, and the standard errors of the mean are presented with error bars. (B) YL2 does not increase transcription or translation of pRSVCAT. pCMVYL2 (9 μg) and pCMVαYL2 (9 μg), which direct the synthesis of YL2 and antisense YL2, respectively, were cotransfected with pRSVCAT (2 μg) into CV1 cells. pRSVCAT was chosen because Rev was expressed from the Rous sarcoma virus promoter in pDM121. The bars represent CAT activities of three transfections, where the standard error of the mean was less than 20%.

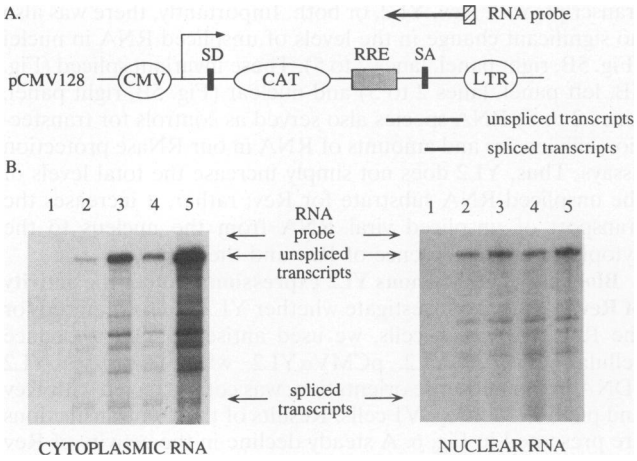

FIG. 5. YL2 increases levels of unspliced viral RNA over those produced by Rev alone in the cytoplasm of CV1 cells. Presented are results of RNase protection assays of transcripts which contain the RRE. (A) Diagrammatic representation of target plasmid pCMV128 and the RNA probe. The cytomegalovirus (CMV) promoter was linked to the *env* region of HIV-1, where the CAT reporter gene was inserted between the splice donor (SD) and the RRE, followed by the splice acceptor (SA) and the HIV-1 long terminal repeat and polyadenylation signal (LTR). A 587-nucleotide-long RNA probe protected 527-nucleotide-long unspliced and 317-nucleotide-long spliced transcripts (below the plasmid) in RNase protections assays. (B) Cotransfections of RRE target plasmid pCMV128 (1 μg) with the effector plasmid(s) coding for Rev (pDM121; 2 μg) and/or YL2 (pCMVYL2; 9 μg) into CV1 cells. Cytoplasmic and nuclear RNAs were extracted as described in Materials and Methods. The left and right panels contained cytoplasmic and nuclear RNAs, respectively. Lanes: 1, mock-transfected CV1 cells; 2, transfections with pCMV128 only; 3, cotransfections with pCMV128 and pDM121; 4, cotransfections with pCMV128 and pCMVYL2; 5, cotransfections with pCMV128, pDM121, and pCMVYL2. All transfections were balanced by addition of pSVCP or pCMV, which contain no target or effector sequences, to avoid promoter competition.

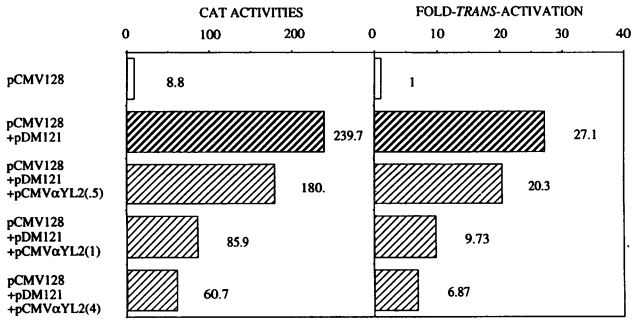

FIG. 6. Antisense YL2 inhibits effects of Rev on the RRE. Increasing amounts (in parentheses after αYL2) of pCMVαYL2, which transcribes YL2 in the antisense orientation, were cotransfected with pDM121 (2 μg) and pCMV128 (1 μg) into CV1 cells. Total DNA was balanced to 11 μg by pCMV in each transfection. CAT activities and fold *trans* activation were measured as described in the legend to Fig. 4. Results are representative of three experiments, where the standard error of the mean was less than 30%.

via 12 transactivation response regions (TARs) and four operators of MS2 (37, 45). As shown in Fig. 7, YL2 increased the activities of these two hybrid effectors on their RNA targets five- and twofold, respectively. However, YL2 had no effects on these RNA targets in the absence of Rex and hybrid Tat-Rev and Rev-MS2 proteins (data not presented). We conclude that YL2 not only potentiates the activities of all known primate retroviruses that contain Rev-like proteins but also increases their effects via heterologous RNA-tethering mechanisms.

DISCUSSION

Using the yeast two-hybrid system, we cloned a mouse protein which binds to Rev of HIV-1. This factor (YL2) contains a high degree of homology (92% identity) to the human 32-kDa protein (p32), which was first identified because it copurifies with alternative splicing factor SF2/ASF (35). Interestingly, YL2 or p32 (YL2/p32) interacts with the basic domain of Rev, which is important not only for its binding to the RRE but also for its effects on RNA splicing in vitro (32, 33). Indeed, even when Rev is targeted to viral RNAs via a heterologous RNA-binding protein (MS2), a basic sequence is still required for the optimal function of Rev (45, 50). Thus, it is not surprising that YL2 also potentiated the activities of Rex on the XRE and the hybrid Tat-Rev and Rev-MS2 proteins on their respective RNA targets (Fig. 7).

Functionally, YL2 satisfies many criteria for the cellular cofactor of Rev. Not only were its effects dependent on Rev and the RRE in cotransfection assays, but the antisense YL2 inhibited *trans* activation by Rev in a dose-dependent manner. However, since YL2 interacts with the basic domain of Rev and the activation domain of Rev has been mapped to its C-terminal leucine residues, it is possible that two different cellular proteins interact with Rev and that YL2 and this other protein complement each other. Indeed, eukaryotic initiation factor 5A binds to the activation domain of Rev and its antisense transcripts block the activity of Rev (47). Alternatively, since these leucines are conserved only in primate lentiviruses and the activation domains of functionally equivalent Revs of equine infectious anemia virus and feline immunodeficiency virus consist of polar residues near their N termini (13, 43), it is also possible that the activation domains of Revs are structural elements that allow for proper folding

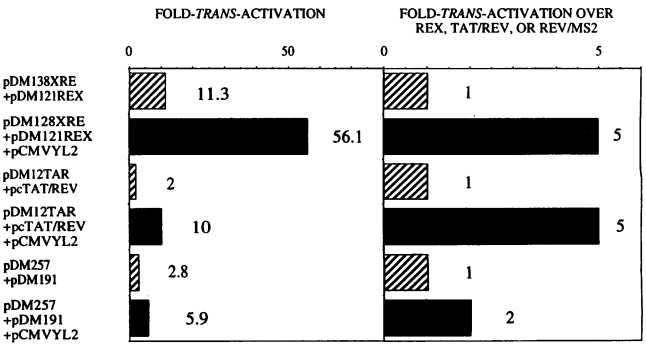

FIG. 7. YL2 potentiates activities of Rex on the XRE of human T-cell leukemia virus type 1 and of hybrid Tat/Rev and Rev/MS2 proteins on 12 TARs and four operators of MS2. Experiments were performed as described in the legend to Fig. 4. However, only data on fold *trans* activation are presented. pDM138RXE, pDM12TAR, and pDM257 contain the XRE, 12 TARs, and four operators of MS2 instead of the RRE. pDM121REX, pcTAT/REV, and pDM191 direct expression of the Rex, hybrid Tat/Rev, and hybrid Rev/MS2 proteins, respectively. YL2 did not activate these targets in the absence of the appropriate Rex or hybrid Tat/Rev or Rev/MS2 protein. The results shown are representative of two experiments, where the standard error of the mean was less than 25%.

and multimerization of Revs on the RRE. Whatever the outcome of these other possible scenarios, YL2/p32 is a very attractive candidate protein for a cofactor of Rev.

p32 is a nuclear protein that binds tightly to p33, which is alternative splicing factor SF2/ASF (35). Since p32 is an acidic protein, it has been proposed that basic residues of SF2/ASF and acidic residues of p32 are critical for their protein-protein interactions (35). Such electrostatic charges might also be responsible for interactions between Rev and YL2/p32. SF2/ASF is a member of a growing family of proteins which share N-terminal ribonucleoprotein-type RNA-binding and serine-arginine-rich motifs that are required for RNA splicing (51). High levels of SF2/ASF promote splicing to proximal 3′ splice sites in vitro (15, 16, 34, 44). In part, this effect might be due to the increased stability and binding of U1 at all splice sites by SF2/ASF (8). More recently, Fu has demonstrated that serine-arginine-rich proteins including SF2/ASF can commit pre-mRNAs to the splicing pathways (14). In his system, SC35 and SF2/ASF committed β-globin and HIV-1 RNAs, respectively, to splicing. Interestingly, the splice donor site in HIV-1 he examined was the same as that present in our target plasmids (14). Given the proposal that Rev functions to "decommit" pre-mRNAs from the splicing pathways and thus promote their export to the cytoplasm (3, 4, 20, 36), it is an intriguing possibility that Rev might function via YL2/p32 to influence the commitment activity of serine-arginine-rich proteins such as SF2/ASF.

YL2/p32 could also act to promote RNA export more directly. If it also interacted with the nuclear pore complex, YL2/p32 could move incompletely spliced viral RNAs out of the nucleus. Alternatively, YL2/p32 could interact with Rev and the splicing machinery and eukaryotic initiation factor 5A would transport these large RNA-protein complexes into the cytoplasm (47). These large mixed complexes would be expected not only to inhibit splicing but also to stabilize the unspliced RNAs. In support of this last notion is the accumulating evidence that unspliced viral transcripts are stabilized by Rev in the nucleus (40).

Thus, YL2/p32 could satisfy all previously described phenotypes for Rev. By binding to the basic domain of Rev, YL2/p32 could mediate the inhibition of RNA splicing by Rev in vitro (32, 33). By interacting with the spliceosome or the transport machinery, YL2 could also facilitate the transport of unspliced viral transcripts from the nucleus to the cytoplasm. Since no increased synthesis of RNA precursors was observed in the nuclei of transfected cells (Fig. 5B), our RNA data confirm this role of YL2. Finally, although all effects of YL2 were documented with transient expression assays, our observations should hold for the live virus. First, we obtained similar results with YL2/p32 in Rev assays by using target plasmids which contain *gag* sequences of HIV-1 (data not presented; 9, 10). Second, our transient expression system has had general applicability for the study of other lentival Rev proteins, for example, those of equine infectious anemia virus and visna virus, in addition to Rev and Rex (23–26, 45). Finally, as an intriguing extension of our studies, antiviral therapies using YL2/p32 might be contemplated in the future.

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