for 10 min in 5% TCA, and washed for 1 min each in 5% cold TCA, ethanol, ethanol-ether (1:1, v/v), and ether. They were dried, and the retained radioactivity was measured. The second method also detected oligopeptides of less than 3 to 4 residues and aa-tRNA bound to ribosomes. Nitrocellulose filters (Sartorius, Long Island, NY; SM 11306, 0.45 μM) were washed twice with 2 ml of standard buffer and dried, and the radioactivity retained was measured. In these experiments the average chain length was at least 20 residues; the stoichiometries obtained with these methods were similar (Figs. 2 and 3). The chain length was calculated from the $^{3}H/^{14}C$ ratio obtained with poly(U)-programmed ribosomes that bound *N*-acetyl[^{14}C]Phe-tRNA^{Phe} in the P site and incorporated [^{3}H]Phe-tRNA^{Phe} in the elongation steps. Inorganic phosphate (P_i) liberated from XTP or GTP was measured by the charcoal method. The reaction, stopped with one volume of 1 M $HClO_4$, 20 mM KH_2PO_4 was diluted tenfold with a 6% charcoal solution in 1 N HCl and centrifuged for 10 min, and the radioactivity in an aliquot was determined (*18*). [γ-^{32}P]XTP was prepared according to I. M. Glynn and J. B. Chappell [*Biochem. J.* **90**, 147 (1964)].

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RBP9-27 protein

```
                                                    40
MHKEEHEVAV LGAPPSTILP RSTVINIHSE TSVPDHVVWS

                                                    80
LFNTLFLNWC CLGFIAFAYS VKSRDRKMVG DVTGAQAYAS

                                                   125
TAKCLNIWAL ILGILMTIGF ILSLVFGSVT VYHIMLQIIQ EKRGY
```

GST–RBP9-27 hybrid protein

```
GST--CTG GTT CCG CGT GGA TCC CCG GGC CAG AAG ATG--RBP927
     leu val pro arg gly ser pro gly gln lys met
                      ↑
     Thrombin cleavage site
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Fig. 1. Amino acid sequences (*27*) of RBP9-27 and of the GST–RBP9-27 recombinant protein expressed in bacteria. The first 28 amino acids of RBP9-27 (underlined) are missing from the β-gal–RBP9-27 hybrid protein produced in λ clone 11. The sequence of the insert in clone 11 begins with Ser^{29} and consists of the 97 COOH-terminal amino acids of the RBP9-27 protein. For bacterial expression, 9-27 cDNA was linked to the GST gene and expressed in pGEX-2T. The hybrid protein was purified and digested with thrombin, resulting in RBP9-27 with six additional amino acids at the NH_2-terminus.

Inhibition of Rev-Mediated HIV-1 Expression by an RNA Binding Protein Encoded by the Interferon-Inducible 9-27 Gene

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Interferon inhibits expression of human immunodeficiency virus type–1 (HIV-1) through unknown mechanisms. A gene inducible by interferon-α (IFN-α) and interferon-γ (IFN-γ) was isolated by screening of a human complementary DNA library for proteins binding to the Rev-responsive element (RRE) of HIV-1. The product of this gene, RBP9-27, was shown to bind RNA in vitro and to inhibit HIV-1 expression after transfection into human cells. RBP9-27 primarily inhibited Rev-dependent posttranscriptional steps of viral gene expression. Thus, RBP9-27 is a cellular factor that antagonizes Rev function. These results suggest an interferon-induced antiviral mechanism operating through the induction of RNA binding proteins such as RBP9-27. Elucidation of RBP9-27 function may lead to a better understanding of the mechanism of interferon action during HIV-1 infection.

Interferons induce a large number of genes in either a direct or an indirect fashion (*1, 2*). The products of these genes either singly or coordinately mediate the antiviral, growth inhibitory, or immunoregulatory activities of interferons. The antiviral effects of interferons against HIV-1 are well established (*3*). Variable amounts of interferon production have been reported in HIV-infected individuals (*4, 5*). Clinical trials of interferon in acquired immunodeficiency syndrome (AIDS) patients are being conducted on the basis of the inhibitory effects of interferon in tissue culture (*6*). Further understanding of the mechanisms of interferon-mediated HIV-1 inhibition may lead to the optimization of the antiviral effects of interferon.

During a screening for cellular proteins that bind to the RRE of HIV-1, we identified an RNA binding protein that inhibits HIV-1 expression. RRE is the site of interaction of the HIV-1 protein Rev with the viral mRNA (*7*). RRE is composed of approximately 210 nucleotides; is located in the *env* coding region of HIV-1, and displays a complex structure important for Rev binding and function (*8, 9*). Rev is required for the expression of structural proteins and thus viral particle formation. Rev protein promotes HIV-1 expression by participating in complex posttranscriptional regulatory steps that involve both positive and negative elements. Rev counteracts the down-regulatory effects of cis-acting HIV-1 sequences (*10–12*) that decrease mRNA transport, stability, and use (*9, 10, 13, 14*). Additional cellular factors are important for Rev function (*15, 16*).

To identify cellular proteins that bind to RRE RNA, we used a 330-nucleotide RRE RNA probe, RRE330 (*15*), uniformly labeled with [^{32}P]uridine triphosphate (UTP) to screen a λ-ZAP XR-II cDNA expression library derived from RNA of the human U937 monocyte cell line (*17*). Twelve recombinant phages were isolated that expressed proteins binding to the RRE probe and not to other RNAs, such as the Tat binding site (TAR, known to form a strong secondary structure) or a globin RNA fragment. We also assessed the binding of the λ clones to different mutated forms of RRE (RREΔ345, RREΔ12s, and RRE220) (*15, 17*). One clone (clone 11) bound to the intact RRE (RRE330), RRE220, and RREΔ12s but did not bind to RREΔ345. This suggested that the recombinant protein produced by clone 11 bound to the region of the RRE containing hairpin loops 3, 4, and 5. The 850-nucleotide cDNA insert of clone 11 contained an open reading frame of 97 amino acids, positioned in-frame with β-galactosidase in λ-ZAP (Fig. 1). A computer search of the GenBank–European Molecular Biology Laboratory (EMBL) database revealed complete identity with the human interferon-inducible gene 9-27 (*18*). The 9-27 gene encodes a protein of 125 amino acids. The cDNA insert in clone 11 encodes the COOH-terminal 97–amino acid residues of this protein, starting at Ser^{29} (Fig. 1). Because our experiments show that the product of the 9-27 gene is an RNA binding protein, we refer to it as RBP9-27.

To express the protein in bacteria, we generated a cDNA fragment of 555 bp after

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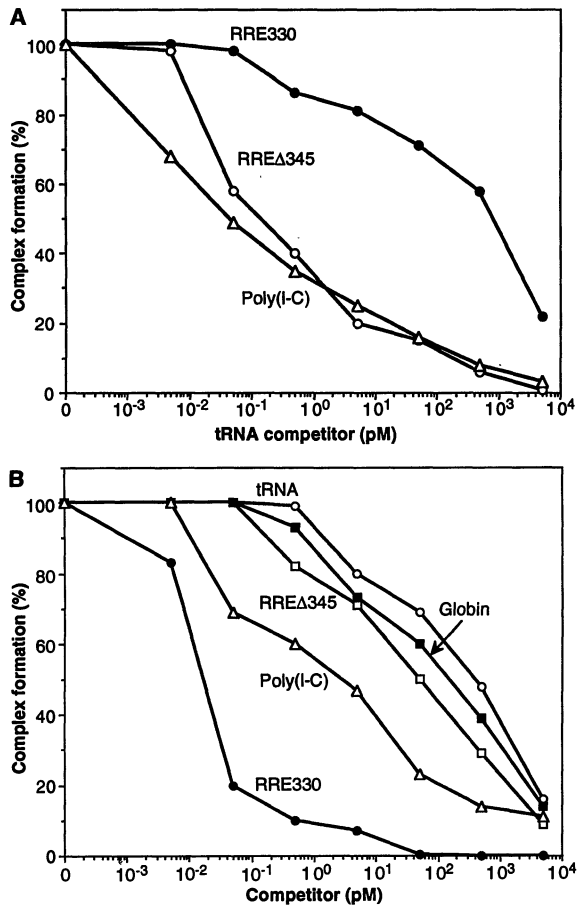

Fig. 2. Specific binding of RBP9-27 to RRE330 RNA. (**A**) Competition of binding of RBP9-27 to labeled RRE330, RREΔ345 RNA, or double-stranded poly(I-C) RNA in the presence of increasing amounts of unlabeled *Escherichia coli* tRNA. The values shown represent averages of triplicate determinations. The radioactivity bound in the absence of competing tRNA was taken as 100%. The SD at each point was less than 10% of the indicated value. (**B**) Competition of RBP9-27 binding to [^{32}P]UTP-labeled RRE330 RNA by various unlabeled nucleic acids. RBP9-27 was bound to labeled RRE330 RNA (*21*) in the presence of increasing amounts of the following unlabeled competitor nucleic acids: RRE330 RNA, RREΔ345 RNA, globin RNA, *E. coli* tRNA, or double-stranded RNA [poly(I-C)].

reverse transcription of RNA from interferon-induced HeLa cells (*18*), followed by polymerase chain reaction (PCR) amplification with 9-27–specific primers (*19*). This cDNA fragment was then subcloned into the bacterial expression vector pGEX-2T (*20*), resulting in plasmid pGST–RBP9-27, which produces a glutathione S-transferase (GST) RBP9-27 fusion protein (GST–RBP9-27). After induction with isopropyl-1-thio-β-D-galactoside (IPTG), we purified GST–RBP9-27 by binding the protein to glutathione-agarose beads and then cleaved it with thrombin to yield RBP9-27 with six additional amino acids at the NH_2-terminus (Fig. 1).

We compared the binding affinity of the bacterially produced RBP9-27 protein for RRE330, RREΔ345, and other RNAs, using an in vitro binding assay in the presence of increasing amounts of tRNA as a competitor (*21*). Binding to RREΔ345 was decreased by 50% in the presence of tRNA (20 ng/ml), whereas ~10^4-fold more tRNA was required for 50% inhibition of binding to RRE330 (Fig. 2A). This is in agreement with the results of the initial screening of λ colonies. We next determined the specificity of binding of RBP9-27 using labeled RRE330 RNA in the presence of increasing amounts of various RNA competitors, such as intact RRE330, RREΔ345, tRNA, globin RNA, and double-stranded poly(I-C) RNA (Fig. 2B). RRE330 competed efficiently for complex formation, whereas a 10^4- to 10^5-fold excess of RREΔ345 or tRNA, respectively, was required for 50% inhibition of RRE330–RBP9-27 complex formation. Double-stranded RNAs are potent activators of the IFN response. Neither poly(I-C) nor poly(A-U) RNA (*22*) bound as tightly to RBP9-27 as did RRE330. No binding of RBP9-27 to double-stranded or single-stranded DNA was detected (*22*). These data demonstrate that RBP9-27 binds preferentially to RRE330 RNA and suggest a role for RBP9-27 protein in HIV-1 replication.

To express the authentic protein in human cells, we subcloned a PCR-amplified, 553-bp 9-27 cDNA into a eukaryotic expression vector between the HIV-1 long terminal repeat (LTR) promoter and the simian virus 40 (SV40) polyadenylation site, thus generating plasmid pL927 (*23*). The production of RBP9-27 in human cells was verified by transfection experiments, followed by immunoprecipitation of the protein by a rabbit serum raised against the purified, bacterially synthesized GST–RBP9-27 fusion protein (Fig. 3A). Cells transfected with pL927 produce a protein of about 17 kD that was specifically recognized by the antiserum to RBP9-27 (anti–RBP9-27). This protein comigrated with the endogenous form of RBP9-27 produced in IFN-induced HeLa cells.

To evaluate the effects of RBP9-27 on HIV-1 expression, we cotransfected the proviral clone pHXB2 with increasing amounts of the RBP9-27–producing plasmid pL927 into a HeLa-derived cell line that constitutively produces HIV-1 Tat (HLtat) (*24*) and measured the production of HIV-1 proteins and RNA. The presence of RBP9-27 resulted in a dose-dependent decrease in production of Env (Fig. 3B) and Gag proteins (Fig. 3, B and C). In contrast, the expression of the cotransfected luciferase-producing plasmid [pRSVluc, containing the Rous sarcoma virus (RSV) promoter] was only marginally affected by RBP9-27 (Fig. 3C). Expression of additional control plasmids, such as pSV2CAT and pCMVCAT, containing the SV40 or the human cytomegalovirus (CMV) promoter next to the chloramphenicol acetyltransferase (CAT) gene, respectively, was not affected by pL927, indicating that the observed inhibition was specific. Two different frame-shift mutations within the 9-27 coding region of pL927 of 20 or 35 NH_2-terminal amino acids of RBP9-27, respectively, did not inhibit HIV-1 expression (*22*). An additional RBP9-27 expression vector containing the CMV promoter (pCMV927) instead of the LTR promoter also inhibited HIV-1 expression (*22*).

To understand the mechanism of inhibition, we analyzed the amounts of viral mRNAs in parallel experiments. Hybridization of Northern (RNA) blots with an HIV-1 LTR probe that detects all three classes of HIV-1 RNAs revealed that the presence of RBP9-27 reduced the amounts of unspliced and intermediate-spliced mRNAs (Fig. 3D). This subset of mRNAs encodes the late viral proteins, including Gag and Env, and requires Rev for expression. The presence of RBP9-27 had less prominent effects on the amounts of the small, multiply spliced viral mRNAs encoding the regulatory proteins that do not require Rev for expression or on the cellular β-actin mRNA (Fig. 3D, bottom). Rehybridization of the blot with a RBP9-27–specific probe confirmed the presence of increasing amounts of RBP9-27 mRNA in the different samples (Fig. 3D, bottom).

Inhibition of the expression of viral structural proteins was also observed with a subgenomic *gag* expression plasmid pCgagA2, which contains the HIV-1 *gag* gene and the RRE flanked by the LTRs (*13*). The pCgagA2 plasmid was analyzed after cotransfection with a Rev-producing plasmid (pL3crev) and a 1-, 5-, 10-, and 20-fold excess of pL927 (Fig. 4A). The presence of increasing amounts of RBP9-27 led to a significant decrease in Gag production,

whereas expression of the cotransfected RSV-luciferase plasmid was only marginally affected. To further analyze the effects of RBP9-27 on Rev function, we studied two additional Gag-expressing plasmids, p37M1234 and p37M1234R, which express the $p37^{gag}$ protein of HIV-1 in a Rev-independent fashion. The p37M1234 plasmid contains several point mutations within the *gag* coding region that alleviate the requirement for Rev protein (*10*, *11*), whereas p37M1234R contains the RRE element as well as the p37M1234 mutations. In contrast to the results obtained with the Rev-dependent plasmid pCgagA2, the presence of increasing amounts of RBP9-27 did not significantly inhibit expression of Gag protein from p37M1234 and p37M1234R (Fig. 4A). These data suggested that the presence of RBP9-27 in trans and of RRE in cis does not inhibit Rev-independent protein expression. This conclusion was further verified by transfection experiments with a plasmid that contains the RSV $p19^{gag}$ gene and RRE linked to the CMV promoter (pCMV-19R, Fig. 4B, bottom). Cotransfection of increasing amounts of pL927 did not significantly affect $p19^{gag}$ RNA or protein expression (Fig. 4, B and C). These experiments established that RBP9-27 inhibits Rev-dependent gene expression by interfering with Rev function.

RBP9-27 belongs to the human 1-8 family of genes that are highly inducible by both type I (α and β) and II (γ) IFNs (*1*, *25*). The family consists of at least three functional genes (9-27, 1-8U, and 1-8D) and one or more pseudogenes (*26*). Examination of the amino acid composition of RBP9-27 and the other members of this family revealed a completely conserved basic region KSRDRK [amino acids 62 to 67 in RBP9-27 (Fig. 1) (*27*)] that may form part of an RNA binding site. In addition, all members contain a potential leucine zipper region close to the COOH-terminus and also contain potential phosphorylation sites (*26*). A region of 45 amino acids within the three proteins [ami-

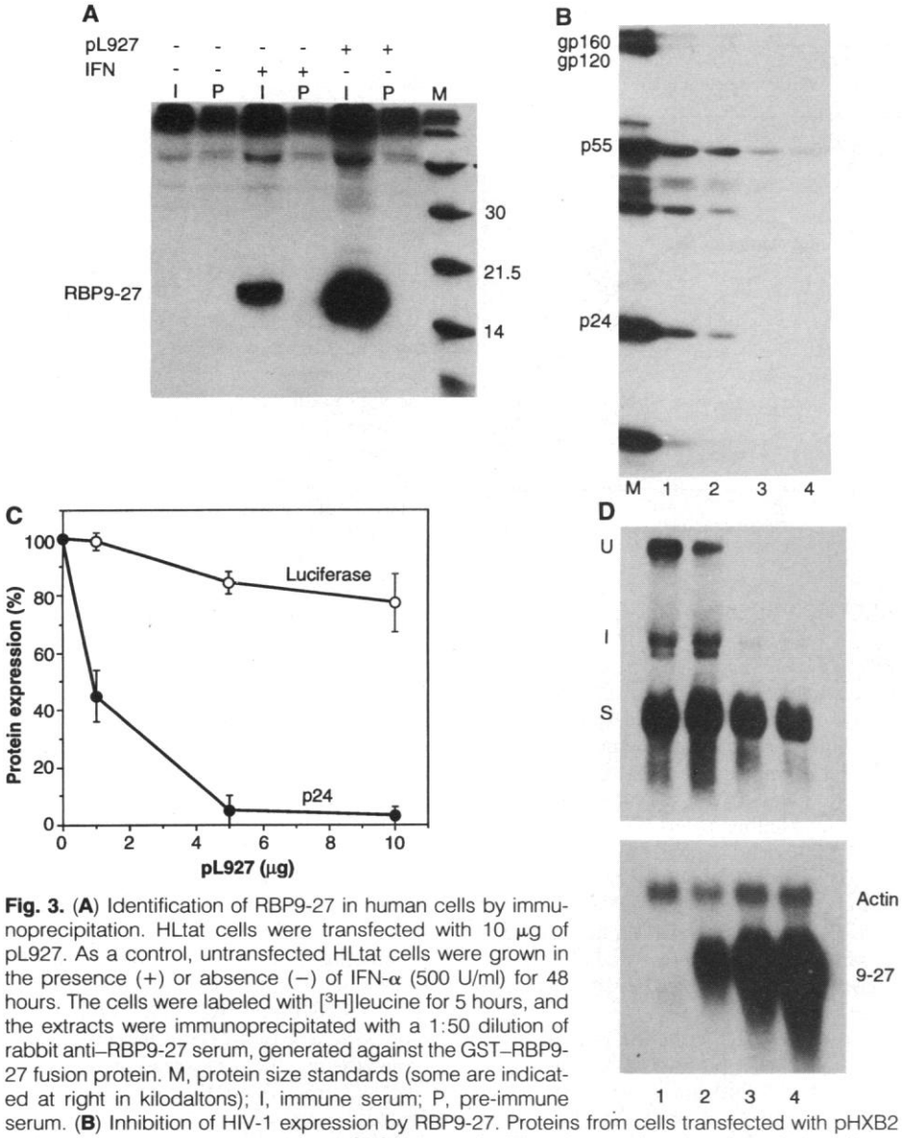

Fig. 3. (**A**) Identification of RBP9-27 in human cells by immunoprecipitation. HLtat cells were transfected with 10 μg of pL927. As a control, untransfected HLtat cells were grown in the presence (+) or absence (−) of IFN-α (500 U/ml) for 48 hours. The cells were labeled with [^{3}H]leucine for 5 hours, and the extracts were immunoprecipitated with a 1:50 dilution of rabbit anti–RBP9-27 serum, generated against the GST–RBP9-27 fusion protein. M, protein size standards (some are indicated at right in kilodaltons); I, immune serum; P, pre-immune serum. (**B**) Inhibition of HIV-1 expression by RBP9-27. Proteins from cells transfected with pHXB2 and pL927 were separated on 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose filters, and probed with serum from HIV-1–infected individuals, followed by ^{125}I-coupled protein A. Lane M contains proteins from HXB2-infected H9 cells; lane 1, pHXB2 alone; lane 2, pHXB2 plus 1 μg of pL927; lane 3, pHXB2 plus 5 μg of pL927; and lane 4, pHXB2 plus 10 μg of pL927. (**C**) Quantitation of Gag protein in the same samples as in (B), measured by the HIV-1 $p24^{gag}$ antigen-capture assay (Coulter). Means ± SD of four independent experiments are shown. The addition of the luciferase-producing pRSVluc serves as a control for transfection efficiency and as an indicator of nonspecific inhibition. Gag or luciferase production in the absence of RBP9-27 was taken as 100%. (**D**) Northern blot of total RNA isolated from transfected cells was hybridized with a random primed, labeled DNA probe spanning the HIV LTR that detects the unspliced (U), intermediate-spliced (I), and small, multiply spliced (S) viral mRNA species. The blot was subsequently washed and rehybridized with probes specific for 9-27 or human β-actin RNAs (bottom). Lanes are as in (B).

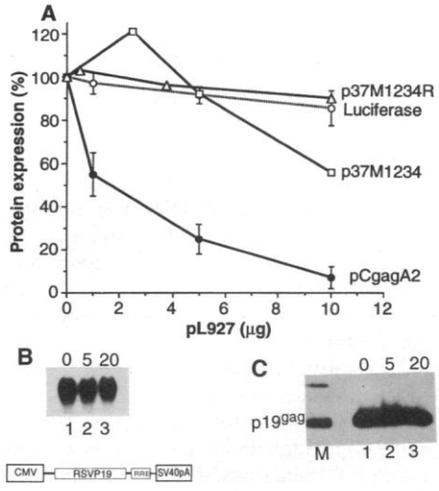

Fig. 4. RBP9-27 inhibits Rev-dependent protein expression. (**A**) Quantitation of Gag production in the presence of RBP9-27 by the $p24^{gag}$ antigen-capture assay. Means ± SD of five independent cotransfection experiments with pCgagA2 and pRSVluc are shown. The effects of RBP9-27 on two additional constructs p37M1234 and p37M1234R, which express high amounts of Gag in the absence of Rev, are also shown. Gag or luciferase production from each plasmid in the absence of RBP9-27 was taken as 100%. (**B** and **C**) HLtat cells were transfected with 5 μg of pCMV-19R [shown at the bottom of (B)] and increasing amounts of pL927 as indicated in micrograms at the top of each lane. One day later, the cells were harvested for RNA (B) and protein (C). Total RNA was isolated and blotted on nitrocellulose, and the blots were hybridized as described with an RSV probe (*11*). Protein immunoblot analysis was performed with an antiserum to $p19^{gag}$. Lane M in (C) contains lysates from RSV-infected cells.

no acids 50 to 94 in RBP9-27 (Fig. 1)] are completely conserved. The amino acid conservation of the three characterized members of the family suggests that they may have similar functions. Using RNA PCR, we cloned the cDNAs of two other members of the family (1-8U and 1-8D) in the same expression vector as described for RBP9-27. Their effect on HIV-1 expression was assayed in similar cotransfection experiments as described for RBP9-27. The product of the 1-8U gene partially inhibited HIV-1 expression, whereas 1-8D had no effect (*22*). These data suggest that the members of the 1-8 gene family express RNA binding proteins with different functional properties and support the conclusion that the function of RBP9-27 on HIV-1 is specific.

The interactions of HIV-1 and other lentiviruses with the IFN system are complex (*28, 29*). Infection of primary cells and cell lines in vitro by HIV-1 does not lead to IFN production. For example, in a survey of 15 different virus isolates, IFN activity was not detected in culture fluids after virus-producing HIV-1 infection (*30*). In fact, there is no evidence for direct IFN induction by any retrovirus in animal or human systems. On the other hand, cocultivation of infected macrophages with peripheral blood mononuclear cells results in IFN induction (*28*), suggesting that specific cellular interactions may be responsible for the induction of IFN in the host after infection. Exogenous IFN has been shown to be a potent inhibitor of HIV-1 expression. Different mechanisms of IFN function against HIV have been proposed (*3, 30, 31*). The production of IFN in HIV-1–infected individuals has been reported and correlates with the development of symptomatic disease (*5, 32*). Peripheral blood mononuclear cells isolated from HIV-1–infected individuals show a diminished ability to produce IFN-α after exposure to viruses.

We have shown that the product of IFN-inducible gene 9-27 (RBP9-27) binds to RRE RNA in vitro and inhibits HIV-1 expression in vivo. Although our experiments suggest different sites of binding of Rev and RBP9-27 with RRE, they may still interfere with each other's function, as has been suggested for Rex and trans-dominant Rev (*8*). These experiments suggest a mechanism for IFN antiviral action through the induction of the RBP-27 family of RNA binding proteins. The studies presented here offer a possible link between IFN-induced antiviral effects and HIV-1 at the molecular level. We propose that one of the ways that the IFN-mediated cellular defense systems inhibit HIV-1 (and possibly other complex retroviruses) is by interfering with the Rev-RRE posttranscriptional regulatory pathway of the virus. Alternatively, it is possible that HIV-1 has developed mechanisms to overcome the negative effects of IFN by inhibiting IFN production or by alleviating the effects of activation of the IFN system. According to this hypothesis, RBP9-27 may have other intracellular functions that lead to HIV-1 inhibition. The binding of RBP9-27 to RRE may be part of a viral strategy to evade the host defenses through binding and inactivating the function of RBP9-27. Viruses fight IFN-mediated inhibition with a variety of mechanisms. For example, adenovirus appears to inhibit interferon response at multiple levels (*33*). Expression of the viral polymerase of hepatitis B virus inhibits the responses of the endogenous 6-16 and 9-27 genes to interferon (*34*). The mechanisms of inhibition of the interferon-inducible genes by different viruses, although not entirely understood, appear to be distinct.

RBP9-27 is a cellular factor that antagonizes Rev function. HIV-1 may use inhibition by IFN to down-regulate its own expression. This may result in the restricted expression of HIV-1 in different cell types. Lentiviral replication strategy involves negative regulatory steps considered important for the generation of chronic, active infections by these viruses (*10, 11*). Further understanding of RBP9-27 function may offer insight into virus-cell interactions and into the development of therapeutic strategies for AIDS prevention.

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17. For library screening, we infected *Escherichia coli* XL-1 Blue bacteria with 8.5×10^5 plaque-forming units of λ-ZAP XR-II U937 cDNA library (Stratagene) and plated them. After 3 to 4 hours at 42°C, the plates were overlaid with nitrocellulose filters soaked in 10 mM IPTG and incubated for 4 to 6 hours at 37°C. Duplicate filters from each plate were denatured with 6 M guanidine-HCl in binding buffer 1 [50 mM tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 10 mM dithiothreitol (DTT)] for 10 min at 4°C. The proteins immobilized on the filters were renatured by sequential lowering of the guanidine-HCl concentration (3 M, 1.5 M, 0.75 M, 0.325 M, and 0.187 M for 5 min each); finally, the filters were equilibrated with binding buffer 1 alone. The filters were incubated in blocking solution (1× Denhardt's in binding buffer) at room temperature for 1 hour, rinsed in binding buffer 1, and hybridized with [^{32}P]UTP-labeled RRE or globin RNAs (0.5 to 1.0×10^6 cpm/ml) in the presence of tRNA (0.1 mg/ml) for 1 hour. After extensive washing, the filters were exposed to x-ray film. Plaques scoring positive with the RRE and negative with the globin probes were identified and picked. After four rounds of plaque purification with the RRE and globin RNA probes, 12 clones were isolated and plated on *E. coli* lawns. The plates were overlaid with IPTG-soaked nitrocellulose filters, and the filters were incubated with labeled RRE330, RREΔ345, RREΔ12s, TAR, or globin RNA probes. The globin RNA probe consists of a 320-nucleotide Sty I–Pst I fragment from the 3′-untranslated region of the human β-globin gene (GenBank/EMBL, file HUHMBB, nucleotides 63862 to 64182). The TAR RNA comprises HIV-1 from nucleotides +1 to +180 and was transcribed from pLG1 [S. Y. Kao, A. F. Calman, P. A. Lucin, B. M. Peterlin, *Nature*,**330**, 489 (1987)]. RRE probes were as described (*15*). RREΔ12s lacks 67 nucleotides comprising hairpin loops 1 and 2 (which contain the Rev binding site), does not bind Rev in vitro, and does not function in vivo; RREΔ345 lacks 82 nucleotides of the RRE comprising hairpin loops 3, 4, and 5; and RRE220 has part of the long stem deleted. RRE330, RREΔ345, and RRE220 have been shown to bind Rev in vitro and to function in vivo (*8, 15*).
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19. To obtain cDNA clones of 9-27, we isolated total RNA from HeLa cells after induction with IFN-α for 48 hours, reverse transcribed the RNA with a mix of random hexamer primers [V. Ciminale, G. N. Pavlakis, D. Derse, C. P. Cunningham, B. K. Felber, *J. Virol.* **66**, 1737 (1992)], and then amplified the cDNA by PCR with 9-27–specific primers. The primers used were 5′-CCCCCGGGCCAGAAGATGCACAAG-3′ (sense) and 5′-TCACAAGCACGTGCACTTTATTGAA-3′ (antisense). The 555-bp amplification product was digested with Sma I and subcloned into Sma I–digested pGEX-2T vector (Pharmacia/LKB) (*20*). The resulting construct (pGST9-27) was electroporated in *E. coli* (JM109). For RBP9-27 purification, bacteria carrying pGST–RBP9-27 were grown to an optical density at 550 nm of 0.7. The production of the fusion protein was induced by addition of IPTG (1

mM final concentration), and the culture was grown for an additional 2 hours. The cells were pelleted and resuspended in buffer A [50 mM tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 5 mM DTT, and phenylmethylsulfonyl fluoride (PMSF; 50 μg/ml)]. After we froze and then thawed the cells, they were lysed by sonication on ice. Triton X-100 and NaCl were added to final concentrations of 1% and 0.3 M, respectively, and the lysate was incubated for 30 min at 4°C on a rotating wheel. The cellular extract was pelleted at 12,000 rpm for 15 min, and the supernatant was dialyzed extensively (4 × 500 ml) against buffer A containing 150 mM NaCl at 4°C. The dialyzed sample was loaded on a glutathione-Sepharose (Pharmacia) affinity column equilibrated with buffer A and washed several times. To cleave the RBP9-27 protein from the GST portion, we washed the column extensively with buffer A containing 2.5 mM $CaCl_2$ but no EDTA or PMSF. The Sepharose beads were then incubated with bovine thrombin (50 μg/ml) in the same buffer for 30 min at room temperature and were washed five times with 1 ml of buffer A to elute the cleaved protein. The thrombin-cleaved RBP9-27 protein was dialyzed against storage buffer [20 mM tris-HCl (pH 8.0), 0.2 mM EDTA, 150 mM NaCl, 5 mM DTT, PMSF (50 μg/ml), and 20% glycerol] and stored at −70°C. We determined RBP9-27 protein to be >95% pure.

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21. RNA-protein complexes were measured by a filter-binding assay. Thrombin-cleaved RBP9-27 (40 pM) was incubated with 0.1 pM of [^{32}P]UTP-labeled RRE330 RNA, RREΔ345 RNA, or poly(I-C) RNA (Pharmacia) in the presence of increasing amounts of unlabeled competitor [*E. coli* tRNA (Boehringer Mannheim) or as specified] in 50 μl of binding buffer 2 [10 mM tris (pH 7.5), 1 mM EDTA, 50 mM NaCl, 2 mM DTT, 0.01% bovine serum albumin (BSA), and RNasin (20 U/ml) for 20 min at room temperature. The complexes were spotted on nitrocellulose filters (25-mm diameter, 0.45-μm pore size; Schleicher Schuell, Keene, NH), washed extensively with binding buffer 2, and air-dried for 30 min. The bound radioactivity was measured in a scintillation counter.
22. P. Constantoulakis *et al.*, unpublished data.
23. Eukaryotic expression plasmids for RBP9-27 and the two related interferon-inducible proteins 1-8U and 1-8D were constructed as follows. Total RNA was isolated from HeLa cells after induction with IFN-α, reverse transcribed, and PCR amplified as described (*19*). The primers used were as follows: 5′-TTCCCCAAAGCCAGAAGATGCA-3′ (sense) and 5′-TCACAAGCACGTGCACTTTATTGAA-3′ (antisense), generating a 553-bp fragment of 9-27 cDNA; 5′-CATGAACCACATTGTGCAAACCT-3′ (sense) and 5′-GAAACATATACACTTTATTGAATG-3′ (antisense), generating a 626-bp 1-8D cDNA; and 5′-CATGAGTCACACTGTCCAAACCT-3′ (sense) and 5′-CCAGAAACACGTGCACTTTAT-3′ (antisense), generating a 563-bp 1-8U cDNA. The PCR fragments were blunt-ended with the Klenow fragment of the DNA polymerase and inserted into Sma I–digested pBluescript KSII (Stratagene). The cDNAs were ligated as Hind III–Bam HI fragments into the Hind III–Bam HI–digested pLdKpA eukaryotic expression vector [B. Mermer, B. K. Felber, M. Campbell, G. N. Pavlakis, *Nucleic Acids Res.* **18**, 2037 (1990)], resulting in pL927, pL18U, and pL18D. The expression vector contains the HIV-1 LTR promoter to +80, a polylinker, and the SV40 polyadenylation signal. HLtat cells were transfected with 5 μg of Gag-expressing plasmid; 1 μg of luciferase expression plasmid (pRSVluc) [J. R. de Wet, K. V. Wood, M. DeLuca, D. R. Helinski, S. Subramani, *Mol. Cell. Biol.* **7**, 725 (1987)]; and 0, 1, 5, and 10 μg of pL927. The total amount of DNA in the transfection mixture was adjusted to 17 μg with carrier DNA. One day later, the cells were harvested for protein or RNA analysis.
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27. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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The Cloning of PIG-A, a Component in the Early Step of GPI-Anchor Biosynthesis

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The glycosylphosphatidylinositol (GPI) anchor is a membrane attachment structure of many proteins and occurs in a wide variety of eukaryotes from yeasts to mammals. The structure of the core of the GPI anchor is conserved in protozoa and mammals and so is its biosynthetic pathway. A complementary DNA encoding a human protein termed PIG-A (phosphatidylinositol glycan–class A) was cloned. PIG-A was necessary for synthesis of *N*-acetylglucosaminyl-phosphatidylinositol, the very early intermediate in GPI-anchor biosynthesis.

More than a hundred eukaryotic cell surface proteins are anchored to the cell membrane by a GPI anchor (*1*). The GPI anchor acts not only as a membrane attachment structure but also as a sorting signal of apically expressed proteins of epithelial cells (*2*, *3*), in a signal transduction mechanism of leukocytes (*4*), as a target of phospholipases that release GPI-anchored proteins (*2*), and in a mechanism that confers increased lateral mobility of membrane proteins (*5*, *6*). Chemical and biosynthetic analyses of the GPI anchor have demonstrated that the core structure of the anchor is conserved in a variant surface glycoprotein of *Trypanosoma brucei* (*7*) and rat T lymphocyte protein Thy-1 (*8*) and that its biosynthetic pathways in protozoa and mammals are similar (*9*–*12*). The molecular cloning of genes encoding enzymes that take part in the biosynthesis of the GPI anchor is necessary to understand the biosynthetic pathway of the anchor and to understand the human disease paroxysmal nocturnal hemoglobinuria, which results from a deficiency of the GPI anchor (*13*).

To clone a cDNA encoding a protein that participates in GPI-anchor biosynthesis, we expressed cDNAs from a HeLa cell library with an Epstein-Barr virus vector (*14*) in the human B lymphoblastoid cell line JY-5, in which synthesis of the GPI anchor is deficient (*15*). Cell lines deficient in GPI-anchor synthesis have been grouped into several complementation classes (*16*), and their biochemical defects have been characterized (*10*–*12*). We analyzed the complementation class of the JY-5 cells by fusing them with murine Thy-1–deficient thymoma cell lines of known classes. JY-5 cells belong to class A that is deficient in an early step of GPI-anchor synthesis (*17*). Transfected JY-5 cells that expressed GPI-anchored proteins were selected by fluorescent flow cytometric sorting (*18*).

After three cycles of sorting and expansion by culture, we identified a cDNA clone

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