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# Specific interaction of the human immunodeficiency virus Rev protein with a structured region in the env mRNA

ALAN W. COCHRANE, CHEIN-HWA CHEN, AND CRAIG A. ROSEN*

Department of Molecular Oncology & Virology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

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**ABSTRACT** **A region of potential complex secondary structure within the human immunodeficiency virus env mRNA has been implicated in Rev-mediated export of viral structural mRNAs from the nucleus to the cytoplasm. By using an RNase protection gel-mobility-shift assay, we demonstrate that purified Rev protein forms a stable complex with this Rev-responsive RNA. RNAs with mutations designed to disrupt formation of a predicted stem structure no longer interact with Rev. However, Rev binding is restored upon annealing of the two complementary RNAs that make up the stem. These results suggest that direct interaction of Rev with the Rev-responsive element could facilitate transport of human immunodeficiency virus structural mRNAs from the nucleus to the cytoplasm.**

The human retroviruses have proven to be an excellent paradigm for the study of the complex mechanisms that govern gene expression. The human immunodeficiency virus (HIV) encodes numerous nonstructural regulatory proteins that influence some facet of viral gene expression and replication. Two of these proteins, referred to as Tat (1, 2) and Rev [formerly referred to as Art (3) or Trs (4)] are essential for virus replication (5–7) and represent potential avenues for therapeutic intervention.

The *rev* gene, which is conserved in HIV-2 (8) and simian immunodeficiency virus (8, 9), encodes a small phosphorylated (10, 11) nuclear protein (12) that accumulates in the nucleolus (13, 14). Synthesis of Rev is required for expression of the viral structural genes (i.e., *gag*, *pol*, and *env*), whereas expression of the nonstructural genes (i.e., *tat*, *vpu*, and *nef*) proceeds in its absence (3, 4, 15). Studies indicate that the block in structural gene expression, in the absence of Rev, results from entrapment of mRNA in the nucleus (16). Although the mechanism that governs this nuclear mRNA sequestration and subsequent Rev-mediated export remains to be determined, results of several independent studies, using different assays to measure Rev function, indicate the presence of "negative" sequences that presumably govern retention of viral mRNA in the nucleus (17–21) and a distinct Rev-responsive element (RRE) (16, 17, 19, 21) that is required for the Rev-mediated export of nuclear mRNA to the cytoplasm. RRE was originally mapped to nucleotides (nt) 7202–7720 (17) and was referred to as CAR for cis-acting antirepression sequence (18). More recent studies have further localized this RRE (16) to near nt 7300–7539, a region that is predicted to have a high degree of secondary structure.

In this study, we show that Rev protein forms a stable complex with RNA containing the Rev-responsive region. Binding requires formation of a stem structure involving the 5′ and 3′ terminal 50 base pairs of the RRE. Mutations that prevent Rev binding also impair Rev responsiveness, suggesting that Rev–RNA complex formation plays an important role in the regulation of HIV gene expression.

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## MATERIALS AND METHODS

**Recombinant Plasmids.** Plasmid constructs utilized in this study were all generated in the Bluescript vector (Stratagene). Numbering of nucleotides is in reference to strain HXBC2 (22). Deletions made within the RRE were prepared by a site-directed mutagenesis protocol described by Kunkel (23). For analysis of Rev protein–RRE RNA interaction, RNA was generated by inserting the RRE or mutations thereof to the 3′ side of the T7 promoter present in the Bluescript vector.

**Purification of Rev Protein.** For expression of Rev, a synthetic Rev gene that contains codons most frequently used in *Escherichia coli* was constructed (24). This synthetic Rev gene was cloned downstream of the $\lambda P_L$ promoter and Rev expression was induced in midlogarithmic phase cultures by shifting the temperature to 42°C. Rev was initially purified from 5 kg (wet weight) of frozen cells as follows. The frozen material was resuspended in 10 liters of 50 mM Tris·HCl (pH 8.0)/1 mM EDTA/5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride, stirred overnight, and then disrupted using a Manton Gaulan apparatus. Cellular debris was removed by a brief centrifugation (10,000 × *g* for 15 min) and the supernatant was adjusted to 0.5% polyethylenimine. Precipitated nucleic acids were pelleted by centrifugation and the coprecipitated Rev protein was released by resuspension of the pellet in 3 M KCl. The resuspended material was then adjusted to 20% (wt/vol) ammonium sulfate and the precipitate, which was found to have the greatest proportion of Rev, was dissolved in isotonic phosphate-buffered saline containing 0.5 M KCl. The mixture was then applied directly to a Sepharose 4B anti-Rev monoclonal antibody immunoaffinity column. The column was prepared using anti-Rev monoclonal antibody at 5 mg/ml of affinity matrix as described by the manufacturer (Pharmacia). Elution and binding conditions were also as suggested by the manufacturer. The anti-Rev monoclonal antibodies were prepared against a synthetic recombinant Rev protein (24) and screened using this protein in an ELISA (L. Tomchak, personal communication).

**Generation of *in Vitro*-Synthesized RNA Transcripts and Rev Binding Assay.** The portion of the HIV *env* gene (nt 7204–7562) encoding the Rev-responsive RNA was cloned downstream of the T7 promoter present in the Bluescript vector. Plasmid DNAs were linearized at a unique *Xba* I site and *in vitro* transcription using T7 RNA polymerase was performed as described by the manufacturer (Promega Biotec). The integrity of the transcribed products was examined by analysis on denaturing 8% polyacrylamide gels.

The mobility-shift RNA-protection assay was carried out as follows. Approximately 50–100 ng of purified Rev protein was resuspended in binding buffer [5 mM Hepes, pH 7.6/25 mM KCl/2 mM $MgCl_2$/3.8% (vol/vol) glycerol] containing 10 μg of nonspecific carrier tRNA. The reaction mixture was preincubated for 5 min at room temperature at which time

Abbreviations: HIV, human immunodeficiency virus; RRE, Rev-responsive element; nt, nucleotide(s).

*To whom reprint requests should be addressed.

labeled RRE RNA (0.5–1 ng, 100,000–200,000 cpm) was added in a final volume of 20 μl. Incubation was continued an additional 15 min at 30°C at which time 5 units of RNase T1 (Pharmacia) was added. Reactions were further incubated at 37°C for 10 min, then adjusted to 10% (vol/vol) glycerol, and applied to a nondenaturing 4% polyacrylamide gel containing 5% glycerol. Electrophoresis was carried out at a constant voltage of 250 V at 4°C in 0.5× TBE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3). For competition experiments tritium-labeled competitor RNAs were added to the incubation prior to addition of the $^{32}$P-labeled RNA.

For those experiments using reannealed RNAs as substrate, the individual RNAs were combined in buffer containing 1 mM EDTA, 50 mM NaCl, and 5 mM Hepes (pH 7.1) at a 2:1 ratio of unlabeled to labeled RNA. The mixture was heated to 90°C for 10 min then slowly brought to room temperature to allow reannealing. The RNAs were diluted in binding buffer and used directly in the Rev binding assay.

## RESULTS

**Rev Forms a Stable Complex with the Rev-Responsive Region.** The minimal region required for Rev response, termed RRE (16), has been mapped to approximately 230 nt spanning nt 7300–7539 in the HIV genome. In our initial series of experiments we examined whether purified Rev protein could form a stable complex with the RRE-containing RNA. As described (24), optimal expression of Rev in *E. coli* can be achieved by creating a synthetic Rev gene that contains codons most frequently used in *E. coli*. For the studies described here, an unmodified form of Rev was purified from *E. coli* using an anti-Rev monoclonal antibody immunoaffinity column. As shown in Fig. 1, a relatively pure fraction of Rev was obtained.

To examine Rev–RNA interactions, an *env* gene fragment, spanning nt 7204–7562, was cloned downstream of the T7 promoter. $^{32}$P-labeled *in vitro*-transcribed RNAs were incubated with purified Rev protein in the presence of 5–10 μg of tRNA, digested with RNase T1, and then analyzed on nondenaturing 4% polyacrylamide gels. RNAs failing to form a complex with the Rev protein will be digested to oligonucleotides and small fragments, while those bound to the protein will be partially protected from the nuclease digestion and remain bound to the Rev protein. As shown in Fig. 2*A*, lane 1, incubation of HIV RNA spanning nt 7204–7562 with Rev resulted in formation of an RNase-resistant RNA–protein complex. The smaller products present after RNase T1 digestion represent regions of secondary structure in the RNA that are resistant to partial RNase treatment.

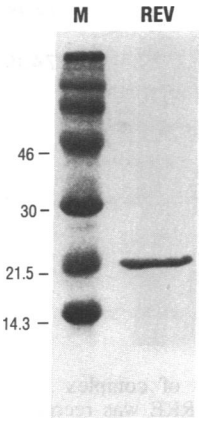


FIG. 1. Purification of Rev from *E. coli*. Rev protein was purified from *E. coli*. An aliquot of the material eluted from the immunoaffinity column was analyzed on a 14% polyacrylamide gel. Protein was visualized by staining with Coomassie blue. Lanes: M, molecular weight markers; REV, Rev protein.

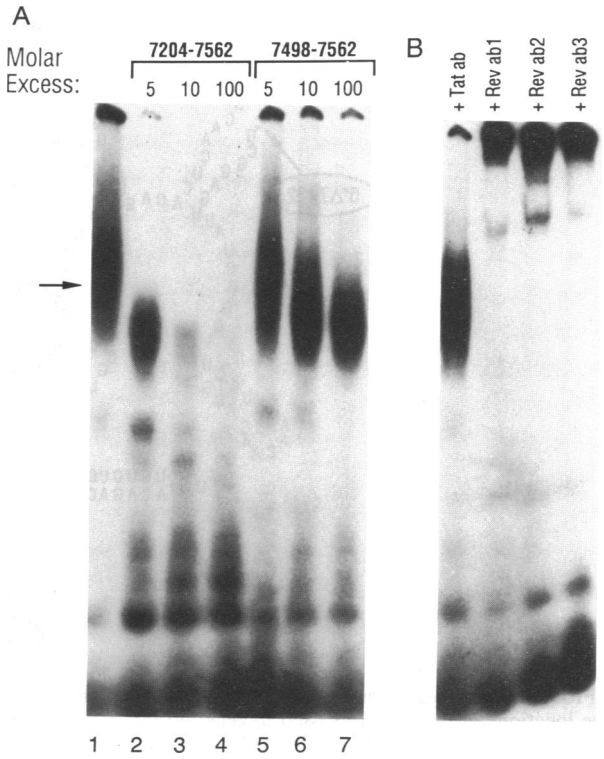


FIG. 2. Complex formation with Rev and RRE RNA. Complex formation was carried out using purified Rev protein and *in vitro*-transcribed RNA corresponding to nt 7204–7562. (*A*) The specificity of complex formation was assessed by preincubation of Rev with the indicated amount of specific (nt 7204–7562) or nonspecific (nt 7498–7562) competitor RNA. The faster migration of the complexes observed in the reactions with competing RNAs is due to the increased amount of salt added to the reaction. Lanes: 1, nt 7204–7562 incubated with Rev; 2–4, nt 7204–7562 incubated with Rev at RNA molar excesses of 5, 10, and 100, respectively; 5–7, nt 7498–7562 incubated with Rev at RNA molar excesses of 5, 10, and 100, respectively. (*B*) The involvement of Rev in complex formation was assessed by including the various monoclonal antibody shown (1 μg) in the reaction.

To examine the specificity of complex formation, reactions were preincubated with an excess of specific RNA competitor (nt 7204–7562) or a nonspecific (see below) competitor (nt 7498–7562) prior to addition of labeled RNA. Unlabeled nt 7204–7562 RNA inhibited complex formation whereas similar quantities of the nt 7498–7562 RNA did not (Fig. 2*A*). Competition with additional deletion RNAs (some of which retain significant secondary structure) that failed to bind *rev* also failed to compete for Rev binding as did the antisense RNA (data not shown).

Although the purity of the preparation of Rev used in these assays would indicate that Rev is responsible for the RNA–protein complexes observed, it was possible that an impurity in the preparation was binding to the RRE-containing RNA. To confirm that Rev itself, rather than a contaminant of the preparations, was responsible for complex formation, reactions were incubated with monoclonal antibodies produced against either the HIV Tat or Rev proteins. Although the anti-Rev antibody did not prevent RNA–protein interaction, the complexes were shifted to the top of the gel, presumably resulting from the formation of high molecular weight antigen–antibody–RRE complex (Fig. 2*B*). No effect was observed with the anti-Tat monoclonal antibody (Fig. 2*B*). These results suggest that Rev protein forms a specific stable complex with the Rev-responsive RNA.

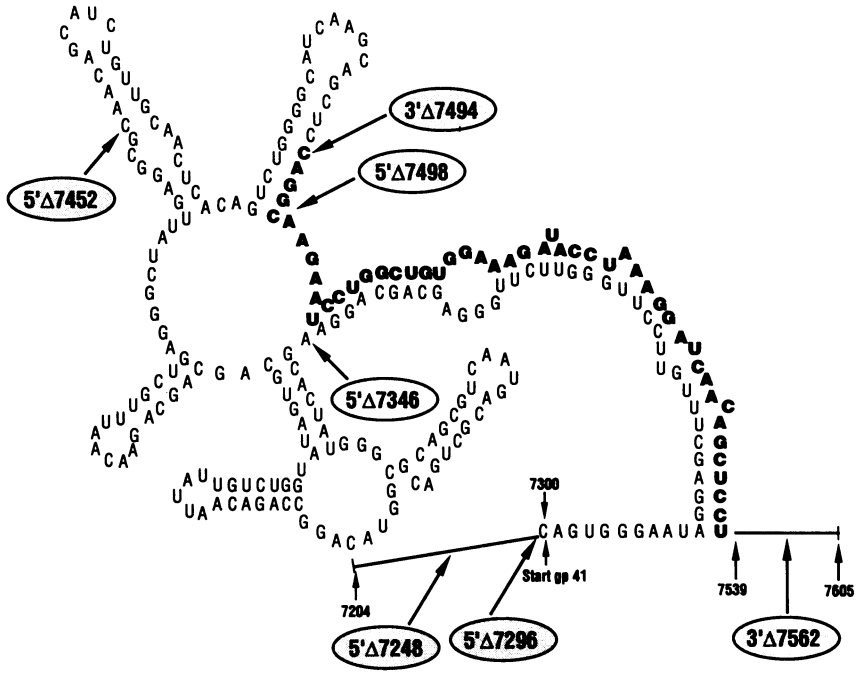


FIG. 3. Predicted secondary structure of the RRE and mutations. The predicted secondary structure (16) assumed by the RRE is shown. The locations of the deletions used to generate the *in vitro*-transcribed RNA are circled. The two RNAs used in the experiments to recreate the RRE structure are indicated by boldface lettering or lightface lettering.

**Rev–RNA Complex Formation Requires the Presence of a RNA with Predicted Secondary Structure.** To examine the sequence requirements for RNA–protein complex formation, a series of deletion mutations were generated in the nt 7204–7605 *env* fragment. The extent of each deletion in the predicted RNA structure is illustrated in Fig. 3. As shown in Fig. 4*A*, Rev–RNA complex formation was unaffected by deletion of nt 7204–7296. However, complex formation was lost as deletions disrupted the predicted stem structure beginning at nt 7300. Addition of a 10-fold excess of the deleted RNAs or an increase in the exposure time of the autoradiogram failed to reveal complex formation (data not shown).

As the deletions entering the 5′ region of the stem structure prevented Rev binding, we reasoned that, if the stem structure is required for Rev interaction, then 3′ mutations designed to disrupt the other half of the stem should give the same result. As shown in Fig. 4*B*, removal of nt 7562–7605 had no affect on complex formation. However, further deletion to nt 7494, which removes the right half of the predicted stem, abolished complex formation.

**Formation of Stem Structure Is Required for Rev Interaction.** The above results strongly suggest that both strands of the large stem in the predicted RRE structure configuration are required for Rev interaction. To confirm this prediction, the stem was recreated by annealing two independently synthesized RNAs that comprise the 5′ and 3′ portions of the stem (Fig. 5*A*). For this experiment, the nt 7204–7494 RNA (Fig. 3, bases in lightface type) was synthesized in the absence of [$^{32}$P]UTP and the semicomplementary nt 7498–

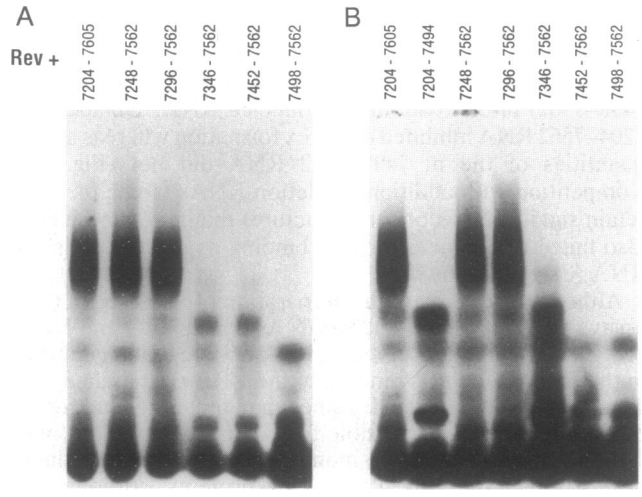


FIG. 4. Sequence requirements for Rev–RNA complex formation. The *in vitro*-transcribed RNAs generated from the deleted plasmids were incubated with Rev and complex formation was carried out. The extent of each deletion in the predicted RRE secondary structure is depicted in Fig. 3. Complex formation with 5′ deletion RNAs (*A*) and complex formation with 3′ and 5′ deletion RNAs (*B*) as indicated are shown.

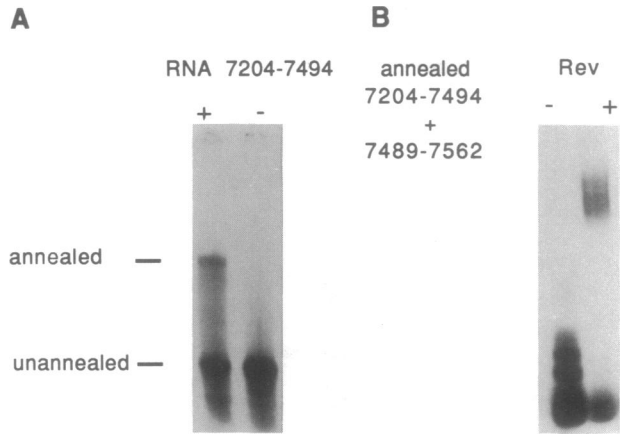


FIG. 5. Restoration of complex formation with reannealed RNAs. (*A*) The entire RRE was recreated by annealing *in vitro*-transcribed RNA spanning nt 7204–7494 (synthesized unlabeled) with one spanning nt 7498–7562 (synthesized with [$^{32}$P]UTP). (*B*) The annealed nt 7204–7494 and nt 7489–7562 RNAs shown were incubated in the presence (+) or absence (−) of Rev and analysis for complex formation was carried out as described for the intact RRE RNA.

7562 RNA (Fig. 3, bases in boldface type) was labeled with $^{32}P$. As shown in Fig. 4, neither of these RNAs could independently form a complex with Rev. However, reannealing of these RNAs (Fig. 5*A*) and incubating with Rev (Fig. 5*B*) restored complex formation. Thus, it appears that the predicted stem structure does indeed form and this region of RRE is critical for Rev interaction.

## DISCUSSION

Previous studies have clearly established that the HIV Rev trans-activator protein functions post-transcriptionally to regulate the transport of the viral structural gene mRNAs from the nucleus to the cytoplasm (16, 19–21, 25). Export of these mRNAs requires the presence of a cis-acting element, termed CAR (18) or RRE (16), present in the env mRNA.

By using purified Rev protein and *in vitro*-transcribed mRNA corresponding to the RRE, we show that Rev forms a specific stable complex with RRE RNA. As the Rev protein used in this study was purified from *E. coli* and consequently lacks post-translational modification, these results indicate that modifications, such as phosphorylation (11, 14), probably do not influence the capacity of Rev to interact with RNA.

By using computer modeling, Malim *et al.* (16) have suggested that the RRE region has the potential to form the complex secondary structure shown in Fig. 3. Although we have not addressed whether the RRE RNA assumes the identical structure depicted, our findings are consistent with formation of the large stem and further suggest that formation of this structure is required for Rev interaction. In support of this conclusion are the following observations: (*i*) mutations within the RRE region that delete all of the stem also abolish Rev binding, and (*ii*) reannealing of individual mRNAs that comprise the stem structure restores Rev binding. We also note that simian immunodeficiency virus and HIV-2, which are responsive to the HIV Rev protein (8), contain an RRE secondary structure and that the most conserved feature with respect to HIV-1 RRE is the stem. Our recent studies indicate that some of the smaller stem loop structures present in the RRE most likely represent the site for Rev interaction (H. Olsen and C.A.R., unpublished data). However, computer modeling suggests that these other secondary structures would not exist without formation of the larger stem structure.

How interaction of Rev with the RRE facilitates transport of mRNA from nucleus to cytoplasm remains to be determined. Possibilities range from the Rev protein functioning as a transporter factor by itself or in combination with another cellular factor in the formation of an mRNP particle. Alternatively, interaction of HIV RNA with Rev may produce a conformational change in the RNA secondary structure that facilitates nuclear transport.

Recent studies indicate that other retroviruses such as HIV-2 (8), simian immunodeficiency virus (9), and human T-cell leukemia virus I (26), share similar complex regulatory pathways. Moreover, the human T-cell leukemia virus I Rex protein, which is believed to function in a manner analogous to Rev (26, 27), shares many common features with Rev and can substitute for Rev in regulation of HIV gene expression (27). Why these viruses have maintained such an elaborate network for the regulation of structural gene expression is not yet clear. One possibility may relate to their cytopathic nature. Perhaps nuclear retention of genomic RNA, which would in turn prevent release of virus and possibly cell death, is maintained until some signal occurs that triggers release. If this is true, one might predict that expression of *Rev* is temporally regulated.

Post-transcriptional regulation of HIV gene expression through interaction of viral trans-activator proteins with secondary RNA structures is probably not limited to the Rev–RRE interactions shown here. A cis-acting sequence, referred to as TAR (28), present in the 5′ untranslated portion of all viral transcripts also forms a complex secondary structure (29) and has been shown (30) to be the binding site for another HIV trans-activator protein, Tat. Further biochemical characterization of the numerous RNA–protein interactions thought to occur with HIV mRNA should shed light on other intriguing mechanisms of post-transcriptional control of gene expression.

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1. Arya, S. K., Guo, C., Josephs, S. F. & Wong-Staal, F. (1985) *Science* **229,** 69–73.
2. Sodroski, J., Patarca, R., Rosen, C., Wong-Staal, F. & Haseltine, W. (1985) *Science* **229,** 74–77.
3. Sodroski, J., Goh, W. C., Rosen, C., Dayton, A., Terwilliger, E. & Haseltine, W. (1986) *Nature (London)* **321,** 412–417.
4. Feinberg, M. B., Jarrett, R. F., Aldovini, A., Gallo, R. C. & Wong-Staal, F. (1986) *Cell* **46,** 807–817.
5. Dayton, A. I., Sodroski, J. G., Rosen, C. A., Goh, W. C. & Haseltine, W. A. (1985) *Cell* **44,** 941–947.
6. Fisher, A. G., Feinberg, M. B., Josephs, S. F., Harper, M. E., Marselle, L. M., Reyes, G., Gonda, M. A., Aldovini, A., Debouk, C., Gallo, R. C. & Wong-Staal, F. (1986) *Nature (London)* **320,** 367–371.
7. Terwilliger, E., Burghoff, R., Sia, R., Sodroski, J., Haseltine, W. & Rosen, C. (1988) *J. Virol.* **62,** 655–658.
8. Malim, M. H., Böhnlein, S., Fenrick, R., Le, S.-Y., Maizel, J. V. & Cullen, B. R. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8222–8226.
9. Colombini, S., Arya, S. K., Reitz, M. S., Jagodzinski, L., Beaver, B. & Wong-Staal, F. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4813–4817.
10. Hauber, J., Bouvier, M., Malim, M. H. & Cullen, B. R. (1987) *J. Virol.* **62,** 4801–4804.
11. Cochrane, A., Kramer, R., Ruben, S., Levine, J. & Rosen, C. A. (1989) *Virology* **171,** 264–266.
12. Cullen, B. R., Hauber, J., Campbell, K., Sodroski, J. G., Haseltine, W. A. & Rosen, C. A. (1988) *J. Virol.* **62,** 2498–2501.
13. Perkins, A., Cochrane, A. W., Ruben, S. M. & Rosen, C. A. (1989) *J. Acquired Immune Deficiency Syn.* **2,** 256–263.
14. Malim, M. H., Böhnlein, S., Hauber, J. & Cullen, B. R. (1989) *Cell* **58,** 205–214.
15. Knight, D. M., Flömerfelt, F. A. & Ghrayeb, J. (1987) *Science* **236,** 837–840.
16. Malim, M. J., Hauber, J., Le, S.-Y., Maizel, J. V. & Cullen, B. R. (1989) *Nature (London)* **338,** 254–257.
17. Rosen, C. R., Terwilliger, E., Dayton, A. I., Sodroski, J. F. & Haseltine, W. A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2071–2075.
18. Dayton, A. I., Terwilliger, E. F., Potz, J., Kowalski, M., Sodroski, J. G. & Haseltine, W. A. (1988) *J. Acquired Immune Deficiency Syn.* **1,** 441–452.
19. Hammarskjold, M.-L., Heimer, J., Hammarskjold, B., Sangwan, I., Albert, L. & Rekosh, D.(1989) *J. Virol.* **63,** 1959–1966.
20. Emerman, M., Vazeux, R. & Peden, K. (1989) *Cell* **57,** 1155–1165.
21. Felber, B. K., Hadzopoulou-Cladaras, M., Cladaras, C., Copeland, T. & Pavlakis, G. N. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1495–1499.
22. Ratner, L., Haseltine, W., Patarca, R., Liuak, K. S., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C. & Wong-Staal, F. (1985) *Nature (London)* **313,** 277–283.
23. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
24. Cochrane, A., Chen, C.-H., Kramer, R., Tomchak, L. & Rosen, C. A. (1989) *Virology* **173,** 335–337.
25. Hadzopoulou-Cladaras, M., Felber, B. K., Cladaras, C., Atha-

nassopoulos, A., Tse, A. & Pavlakis, G. N. (1989) *J. Virol.* **63,** 1265–1274.
26. Hidaka, M., Inoue, J., Yoshida, M. & Seiki, M. (1988) *EMBO J.* **7,** 519–523.
27. Rimsky, L., Hauber, J., Dukovich, M., Malim, M. H., Langlois, A., Cullen, B. R. & Greene, W. C. (1988) *Nature (London)* **335,** 738–740.
28. Rosen, C. A., Sodroski, J. G. & Haseltine, W. A. (1985) *Cell* **41,** 813–823.
29. Muesing, M. A., Smith, D. H. & Capon, D. J. (1987) *Cell* **48,** 691–701.
30. Dingwall, C., Ernberg, I., Gait, M. J., Goren, S. M., Heaphy, S., Karn, J., Lowe, A. D., Singh, M., Skinner, M. A. & Valerio, R. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6925–6929.