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The Rev Protein of Human Immunodeficiency Virus Type 1 Promotes Polysomal Association and Translation of *gag/pol* and *vpu/env* mRNAs

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Biochemical examination of the Rev-dependent expression of *gag* mRNAs produced from *gag*-Rev-responsive element (RRE) expression plasmids showed a large discrepancy between the level of cytoplasmic *gag* mRNA and the produced Gag protein. Significant levels of the mRNA produced in the absence of Rev were localized in the cytoplasm, while very low levels of Gag protein were produced. In the presence of Rev, the levels of mRNA increased by 4- to 16-fold, while the Gag protein production increased by 800-fold. These findings indicated that in addition to promoting nucleus-to-cytoplasm transport, Rev increased the utilization of cytoplasmic viral mRNA. Poly(A) selection and in vitro translation of cytoplasmic *gag* mRNA verified that the mRNA produced in the absence of Rev was functional. To analyze the translational defect in the absence of Rev, we examined the association of the cytoplasmic *gag* mRNA with ribosomes. *gag* mRNA produced in the absence of Rev was excluded from polysomes, while *gag* mRNA produced in the presence of Rev was associated with polysomes and produced Gag protein. These observations showed that the presence of Rev was required for efficient loading of *gag* mRNA onto polysomes. This effect required the presence of the RRE on the mRNA. Analysis of mRNAs produced from a *rev*-minus proviral clone confirmed that the presence of Rev promoted polysomal loading of both *gag/pol* and *vpu/env* mRNAs. The localization of *gag* mRNA was also examined by in situ hybridization. This analysis showed that in the presence of Rev, most of the *gag* mRNA was found in the cytoplasm, while in the absence of Rev, most of the *gag* mRNA was found in the nucleus and in the region surrounding the nucleus. These results suggest that a substantial fraction of the *gag* mRNA is retained in distinct cytoplasmic compartments in the absence and presence of Rev. These findings indicate that the presence of Rev is required along the entire mRNA transport and utilization pathway for the stabilization, correct localization, and efficient translation of RRE-containing mRNAs.

Human immunodeficiency virus type 1 (HIV-1) encodes a nuclear phosphoprotein named Rev (for recent reviews, see references 30, 53, and 57), which is essential for the production of the late viral proteins Gag, Pol, Env, Vpu, Vpr, and Vif (21, 27, 31, 63, 66). These proteins are encoded by unspliced and partially spliced mRNAs which contain a *cis*-acting element termed the Rev-responsive element (RRE) (25) or CAR (16), located in the *env* region (16, 20, 31, 32, 45, 58). RRE spans an element of approximately 204 nucleotides (nt) which is proposed to form a strong secondary RNA structure (12, 17, 35, 43, 67). In the absence of Rev, these mRNAs are preferentially localized in the nucleus, where they are either degraded or fully spliced. Therefore, comparatively low levels of the unspliced and partially spliced mRNAs are found in the cytoplasm. Rev increases the stability (25, 64) and transport of the unspliced and partially spliced mRNAs to the cytoplasm (20, 25, 32, 45), where they are translated efficiently into structural proteins. Subsequent experiments have established that Rev binds specifically to RRE in vitro (6, 12, 14, 15, 34, 35, 43, 50, 51, 71). Mutations introduced in the leucine-rich acidic domain of Rev (amino acids 79 to 88) result in Rev proteins that competitively inhibit the function of Rev in vivo (44, 46). Such transdominant mutant Rev proteins do not themselves affect the steady-state levels of HIV-1 RNA (6). Results of in vitro binding experiments indicate that these proteins exert their inhibitory effect by binding to RRE and preventing Rev-RRE complex formation (6, 50). Therefore, binding to RRE, although necessary, is not sufficient for the expression of the RRE-containing mRNA. Several lines of evidence suggest that cellular factors interacting with Rev are necessary for Rev function (1, 6); transdominant mutant Rev proteins apparently are unable to interact with these cellular factors.

In addition to influencing the fate of the viral mRNA within the nucleus, Rev has been proposed to affect utilization of viral mRNA at the level of translation (6, 31, 66). To investigate this possibility, we have studied the localization and expression of *gag*-RRE mRNAs in further detail. We find a large discrepancy between the level of the cytoplasmic mRNA and the produced protein. Although significant levels of *gag* mRNA were found in the cytoplasm in the absence of Rev, translation of the mRNA into Gag protein was strictly dependent on the presence of Rev. Results of RNA fractionation experiments indicated that Rev promotes the association of RRE-containing mRNAs with polysomes. These findings establish that Rev is required along the entire transport pathway for both the correct localization and efficient translation of the viral mRNA.

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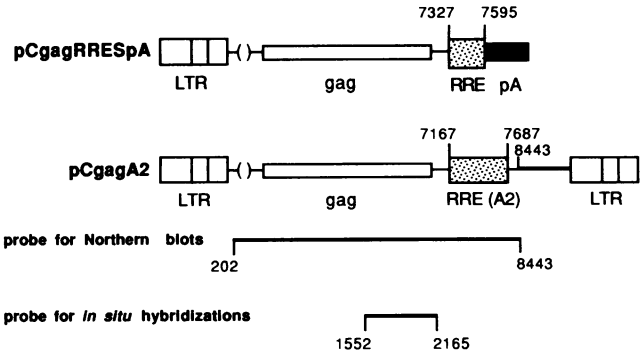

FIG. 1. *gag* expression plasmids. Indicated are the eukaryotic *gag* expression plasmids pCgagRRESpA and pCgagA2 and the probes used for Northern analyses and in situ hybridizations. The parentheses upstream of the *gag* gene indicate the small deletion which removed the 5′ splice site. LTR, long terminal repeat.

MATERIALS AND METHODS

Constructs. All eukaryotic expression plasmids were constructed from HIV-1 clone HXB2 (26). The numbering follows the corrected HXB2 sequence (GenBank accession numbers K03455 and M38432) (47, 54, 55, 67), starting with the 5′ terminus of 5′ R repeat (mRNA start). *gag* expression plasmids were derived from pCgagA2, which contains the following: the HXB2 5′ long terminal repeat promoter and *gag* gene (to the *BalI* site at nt 2165) with a small internal deletion removing the major splice donor sequence at nt 287, the RRE (fragment A2, nt 7167 to 7687), and the 3′ portion of the HIV-1 genome (nt 8443 to 9265) (25). pCgagRRESpA, formerly referred to as pCgagRREpA (6), is similar to pCgagA2 except that it contains a smaller RRE fragment (previously named Sty270; nt 7327 to 7595 [67]) and the polyadenylation signal of simian virus 40 (SpA) in place of nt 8443 to 9265. Northern (RNA) blot analysis using different probes verified that pCgagA2 and pCgagRRESpA did not produce spliced mRNA. pCgagpA was derived from pCgag RRESpA by deletion of RRE. The *rev*-minus proviral mutant fB was constructed by the insertion of 4 nt at the unique *Bam*HI site of HXB2c (21, 31, 66). *rev* expression plasmids pHCMVsrev (6) and pLsrev (46) have been described elsewhere.

Transfections, RNA analysis, and protein assays. Plasmid DNA was prepared and transfected into the Tat-producing cell line HLtat (62) as described previously (24, 29). pRSV-luciferase (18) was included as an internal standard for transfection efficiency. All transfections were done in duplicate for protein and RNA analysis. One day posttransfection, cells were harvested for analysis of protein, total RNA, or nuclear and cytoplasmic RNA. Total RNA was isolated by the heparin-DNase I method (25, 41); nuclear and cytoplasmic RNAs were prepared as described previously (25) and isolated by the hot phenol method (61). Northern blot analysis was performed as described previously (31). *gag* RNA was detected on Northern blots by hybridization to the ^{32}P-labeled 2.6-kb *Sac*I-*Xho*I fragment of pCgagA2 (Fig. 1). β-Actin mRNA was detected on Northern blots by hybridization with the ^{32}P-labeled 2.0-kb *Hin*dIII fragment of pβ-2000 (11). 28S and 47S rRNAs were detected on Northern blots by using ^{32}P-labeled synthetic oligonucleotides, which were complementary to nt 109 to 143 and 578 to 620 of 28S rRNA (28) and nt 1 to 40 and 433 to 451 of 47S rRNA precursor (38). Analysis of RNA by reverse transcription of RNA followed by amplification using the polymerase chain reaction (RT-PCR) was carried out essentially as described previously (3, 59, 60, 62), following DNase I treatment to remove contaminating DNA. Reverse transcription was performed by using pd(N)$_6$ (Pharmacia) as the primer. PCR was performed for 25 cycles (1 min of denaturation at 91°C, followed by 2 min of polymerization at 65°C) as described previously (3) in a Perkin-Elmer Cetus DNA thermal cycler. cDNA synthesized from β-actin pre-mRNA (49) was amplified by using a ^{32}P-labeled sense primer corresponding to an intron E sequence (nt 2837 to 2860) and an antisense primer complementary to an exon 6 sequence (nt 3003 to 3021) to produce a 185-nt amplified product. cDNA synthesized from mature β-actin mRNA was amplified by using a ^{32}P-labeled sense primer corresponding to an exon 5 sequence (nt 2721 to 2741) and the exon 6-specific antisense primer described above to produce a 189-nt amplified product. cDNA synthesized from mRNA produced by the HIV-1 *rev*-minus proviral clone fB was amplified by using a ^{32}P-labeled sense primer corresponding to nt 173 to 197 and an antisense primer complementary to one of the following sequences: nt 455 to 480 in the *gag* gene, nt 5609 to 5630 in the *env* gene, or nt 5382 to 5409 in the *tat* gene. Parallel reactions were performed in the absence of reverse transcriptase to confirm that the DNase I treatment effectively removed contaminating DNA, and RT-PCR was performed on serial dilutions of the RNA to establish the linear range of amplification. Radioactivity on Northern blots and acrylamide gels was quantitated with the AMBIS radioanalytic imaging system. Gag protein production was quantitated by p24^{gag} antigen capture assay (Dupont, NEN) (67); luciferase production was quantitated as described previously (7, 18, 67).

In situ hybridizations. HLtat cells were plated onto glass slides and transfected as described above. One day later, the slides were fixed for 20 min in 4% paraformaldehyde solution, rinsed, dehydrated in ethanol, and stored at −70°C until processed further (4). A duplicate slide from each transfection was processed for indirect immunofluorescence (25) to assess transfection efficiency. In situ hybridizations were carried out essentially as described elsewhere (68), except that the proteinase K step was omited. The method consisted of the following steps: the slides were acetylated, rinsed, dehydrated in ethanol, and hybridized overnight at 52°C with a [^{3}H]UTP-labeled RNA probe. Slides were then rinsed, treated with RNase A and RNase T$_1$ (40 μg/ml each) at 37°C for 1 h, rinsed extensively, dehydrated in ethanol-ammonium acetate, and coated with Kodak NTB2 emulsion. Slides were then exposed, developed, stained with hematoxylin and eosin, dehydrated in ethanol and xylenes, and mounted with coverslips.

RNA probes were generated by using pBS3′gag, which was constructed by insertion of a fragment spanning nt 1552 to 2165 of HXB2c between the T3 and T7 promoters of Bluescript KS− (Fig. 1). The RNA probe was prepared (68) by transcribing the *Bam*HI-linearized plasmid with T3 polymerase in the presence of [^{3}H]UTP. Transcription reactions were treated with DNase RQ1 and subjected to alkaline hydrolysis to obtain 100- to 150-base fragments. A sense probe prepared from *Nco*I-linearized DNA by using T7 polymerase served as a negative control to detect hybridization to DNA.

In vitro translations. Cytoplasmic RNA was prepared, and polyadenylated mRNAs were selected by oligo(dT) chromatography as recommended by the manufacturer (Pharmacia). The polyadenylated mRNAs (0.5 μg) were translated in vitro in the presence of [^{35}S]methionine in a rabbit reticulocyte

lysate system (Promega). Translated products were immunoprecipitated (24) with HIV-1-positive human immune serum known to react with Gag. Proteins were then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (42) and visualized by fluorography (9).

RNA fractionation. For all RNA fractionation experiments, transfections were done in triplicate in 60-mm plates and the extracts were pooled. Cytoplasmic extracts were prepared from the cultures by treatment of the cells directly on the plates (69) as follows. Plates were placed in an ice water bath and rinsed once with 5 ml of ice-cold hypotonic buffer (5 mM Tris-Cl [pH 7.4], 1.5 mM KCl, 2.5 mM $MgCl_2$). The cells were then incubated for 10 min in 1.2 ml of ice-cold hypotonic buffer containing 1% deoxycholic acid and 1% Triton X-100 (extraction buffer). The resulting cytoplasmic extract was removed, and the nuclei, which remain attached to the plate during this procedure, were rinsed with an additional 0.8 ml of extraction buffer; this rinse was combined with the first extract to give a total volume of 6 ml. This combined extract was centrifuged at 500 × *g* and 4°C for 5 min to remove cell debris. RNasin and dithiothreitol were added to the resulting clarified extract to final concentrations of 100 U/ml and 2 mM, respectively; 1.2-ml portions were then applied to each of three 17 to 41% sucrose gradients buffered with 10 mM Tris-Cl (pH 7.4)–85 mM KCl–2.5 mM magnesium acetate and centrifuged at 40,000 rpm and 4°C for 2 h 40 min in an SW40 rotor. Alternatively, the clarified cytoplasmic extracts were immediately frozen as 0.6-ml aliquots in a dry ice-acetone bath, stored at −80°C, and then thawed in an ice water bath when needed. Gradients were fractionated and monitored for A_{254} with an ISCO gradient fractionator and monitor. The absorbance profiles obtained for the different gradients were found to be virtually superimposable (data not shown). Fractions obtained from the sets of gradients were pooled as specified in the figure legends, extracted twice with phenol-chloroform and once with chloroform, and ethanol precipitated. Concentrations of RNA were then determined by spectrophotometry to confirm the presence of RNA, and the samples were subjected to Northern blot analysis or RT-PCR. Prior to RT-PCR, each sample was treated with DNase I to remove contaminating DNA, extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with ethanol in the presence of carrier tRNA. Pellets were resuspended in H_2O, and RT-PCR was carried out as described above and in the figure legends.

RESULTS

Rev-dependent expression of cytoplasmic *gag* mRNA. The effect of Rev on the production of *gag* mRNA and Gag protein was studied by using the *gag* expression plasmid pCgagRRESpA (Fig. 1). This plasmid is a derivative of the previously described *gag* expression plasmid pCgagA2 (25) (Fig. 1) and contains the 5′ LTR, *gag* gene, and RRE, linked to the polyadenylation signal of simian virus 40 (6). pCgagRRESpA does not contain any recognized splice sites and produces only unspliced *gag* mRNA. This plasmid was transfected into a HeLa cell line that constitutively expresses Tat protein (HLtat cells [62]) in the absence and presence of a *rev* expression plasmid. A plasmid expressing luciferase was included in all transfections and served as a control for transfection efficiency (18). Results demonstrated that although significant levels of total *gag* mRNA were detected both in the absence and presence of Rev, production of Gag protein from pCgagRRESpA was strictly dependent on the presence of Rev (see below). Quantitation of the luciferase standard established that this effect was not due to differences in transfection efficiency. The observed Rev-dependent expression of Gag protein from pCgagRRESpA was in agreement with previously published findings made by using various viral deletion mutants, including the parent *gag* expression plasmid pCgagA2 (6, 25, 31).

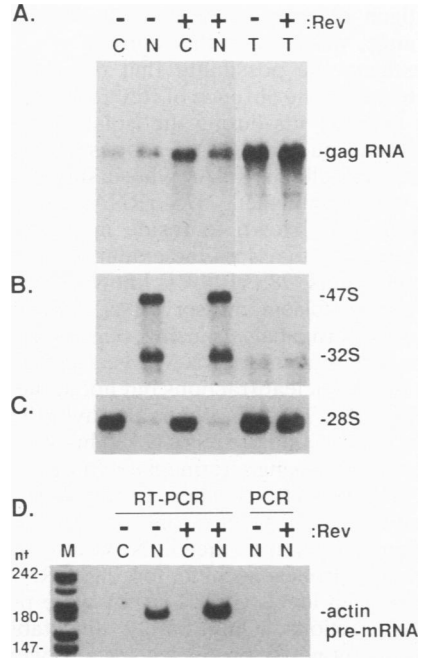

FIG. 2. Subcellular localization of *gag* RNA produced from pCgagRRESpA. (A) Cytoplasmic (C), nuclear (N), and total (T) RNA produced in HLtat cells transfected with pCgagRRESpA in the absence (−) or presence (+) of the Rev-producing plasmid pHCMVsrev was subjected to Northern blot analysis using the *gag*-RRE probe depicted in Fig. 1. (B) A duplicate of the Northern blot shown in panel A was probed with ^{32}P-labeled synthetic oligonucleotides specific for the 47S rRNA precursor. (C) The Northern blot shown in panel A was reprobed with ^{32}P-labeled synthetic oligonucleotides specific for 28S rRNA. (D) Cytoplasmic and nuclear RNA from the same experiment was subjected to RT-PCR to detect the presence of β-actin pre-mRNA. Resulting products were separated on a 6% nondenaturing polyacrylamide gel. Reactions in lanes marked PCR were performed without reverse transcriptase.

The subcellular distribution of the mRNA produced from pCgagRRESpA was studied by separating mRNA into cytoplasmic and nuclear fractions. As shown in Fig. 2A, significant levels of *gag* mRNA were detected in the cytoplasm both in the absence and presence of Rev. In agreement with observations made previously by comparing total *gag* mRNA and Gag protein levels, a consistently large discrepancy existed between the levels of cytoplasmic *gag* mRNA and produced Gag protein. For example, in one experiment, levels of cytoplasmic *gag* RNA and $p24^{gag}$ protein were increased by 4.4- and 845-fold, respectively; in a second experiment, RNA and protein were increased by 16- and 848-fold, respectively. This difference between RNA and produced protein is higher than reported before by our laboratory (6, 25, 31) because of an improvement in specificity and sensitivity of the technique used to quantitate $p24^{gag}$ protein (i.e., Western immunoblot analysis followed by densitometry was used in previous studies, whereas the

p24^{gag} antigen capture assay system, which has a great dynamic range, was used in this study).

To investigate the possibility that the cytoplasmic *gag* mRNA detected in the absence of Rev resulted from leakage of nuclear components during the isolation procedure, the cytoplasmic and nuclear RNA fractions were analyzed for the presence of cellular RNAs whose subcellular distributions are well established. 47S rRNA, the precursor to mature rRNAs, is known to reside in the nucleolus and served as a control to assess the extent of disruption of the nuclear components. 28S rRNA is known to reside predominantly in the cytoplasm and served as a cytoplasmic RNA standard. Northern analysis using oligonucleotide probes specific for 47S precursor rRNA revealed the presence of 47S rRNA in the nuclear fractions but not in the cytoplasmic fractions (Fig. 2B). The 47S probes also hybridized to a band migrating at the position of 32S rRNA. This species probably represented a processing intermediate still containing the 5′ end of the precursor rRNA. These results demonstrated that the nucleoli remained intact during the isolation procedure and partitioned with the nuclei. Northern analysis using oligonucleotide probes specific for 28S rRNA (Fig. 2C) revealed that high levels of 28S rRNA were present in the cytoplasmic fractions. Only barely detectable amounts, likely to represent newly processed species, were present in the nuclear fractions. These observations indicated that the nuclear fractions were not contaminated with cytoplasmic species.

Although the correct distributions of 28S and 47S rRNA argued against artifactual contamination or leakage of nuclear components, these data did not completely rule out the possibility of leakage of mRNAs from the nucleus. Therefore, we determined the distribution of the precursor to β-actin mRNA in the cytoplasmic and nuclear fractions. This was performed by RT-PCR. The primers used were located in the last intron and exon of the β-actin gene. As shown in Fig. 2D, the expected 185-nt amplification product corresponding to the β-actin precursor mRNA was detected only in the nuclear fractions, not in the cytoplasmic fractions. This band also was not detected in reactions in which reverse transcriptase was omitted. This confirmed that the 185-nt band was amplified from cellular pre-mRNA and not from contaminating genomic DNA. Taken together with the results obtained by studying the two rRNAs, these findings allowed us to conclude that the *gag* mRNA detected in the cytoplasm in the absence of Rev did not represent nuclear species that had escaped into the cytoplasm during the fractionation procedure.

In vitro translation of cytoplasmic *gag* mRNA produced in the absence of Rev. We next examined whether the cytoplasmic *gag* RNA detected in the absence of Rev was functional. Cytoplasmic RNA was isolated from HLtat cells transfected with pCgagRRESpA in the absence or presence of Rev, and polyadenylated species were purified by oligo(dT) chromatography. Figure 3A shows a Northern blot analysis of 0.5 μg of poly(A)-selected RNA. *gag* mRNA was detected after poly(A) selection in both the absence and presence of Rev, demonstrating that the mRNA produced in the absence of Rev was polyadenylated.

The translatability of this RNA was determined by using a rabbit reticulocyte lysate in vitro translation system. Translation products were subjected to radioimmunoprecipitation with an HIV-1-positive human immune serum known to react with Gag. As shown in Fig. 3B, p55^{gag} protein was synthesized from both RNA preparations. Comparisons of Northern blots and immunoprecipitations indicated that the

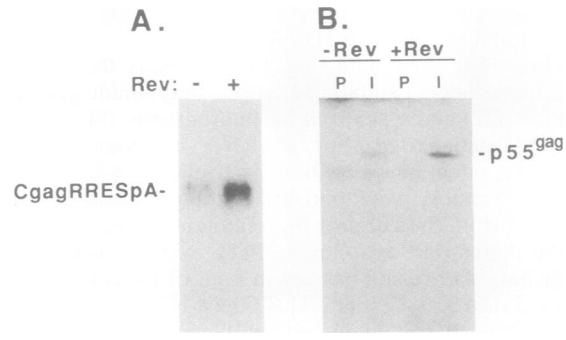

FIG. 3. In vitro translation of *gag* mRNA. (A) Northern blot analysis of poly(A)$^+$ *gag* mRNA. HLtat cells were transfected with pCgagRRESpA, with or without the *rev* expression plasmid pHCMVsrev. Cytoplasmic RNA was prepared, and polyadenylated mRNAs were selected by oligo(dT) chromatography. Shown is a Northern blot of 0.5 μg of the resulting mRNAs, visualized by using the *gag*-RRE probe shown in Fig. 1. (B) Immunoprecipitations of in vitro-translated Gag protein. The polyadenylated mRNAs described above (0.5 μg) were translated in vitro in the presence of [^{35}S]methionine in a rabbit reticulocyte lysate system. Translated products were immunoprecipitated (24) with HIV-1-positive human immune serum (lanes I) or with normal human serum (lanes P) and subjected to SDS-polyacrylamide gel electrophoresis through a 15% gel. The position of p55^{gag} is indicated at the right.

amounts of p55^{gag} produced were proportional to the amounts of *gag* mRNA added to the translation mixtures. The observation that *gag* mRNA produced from pCgagRRE SpA in the absence of Rev was translated in vitro indicated that the mRNA was functional.

Association of *gag* mRNA with polysomes. To study the translational defect at the level of ribosomal loading, cytoplasmic extracts were prepared from cells transfected with pCgagRRESpA in the absence or presence of Rev and subjected to sucrose gradient centrifugation. Figure 4A shows a representative A_{254} gradient profile. Gradient fractions were pooled, treated with DNase I, and subjected to comparative RT-PCR analysis to detect *gag* mRNA in the different fractions. Resulting products were separated on nondenaturing acrylamide gels and visualized by fluorography. Consistent with previous findings, approximately fourfold-higher levels of unfractionated cytoplasmic *gag* mRNA were detected in the presence of Rev (compare lanes C in Fig. 4B and C; also see Fig. 2A). The cytoplasmic *gag* mRNA produced in the absence of Rev was found almost exclusively in the free, subunit, and monosome fractions (Fig. 4B, lanes 1 to 5). Longer exposures of the gel shown in Fig. 4B verified the absence of polysome-associated *gag* mRNA. In contrast, *gag* mRNA produced in the presence of Rev was detected in the free, subunit, and monosome fractions (Fig. 4C, lanes 1 to 5) as well as in the polysome fractions (lanes 6 to 10). Parallel analysis of the gradient fractions in the absence of reverse transcriptase confirmed that the DNase I treatment had been effective in removing contaminating DNA (data not shown).

Failure to detect polysome-associated *gag* mRNA in the absence of Rev was not due to artifactual dissociation of polysomes during the extraction or centrifugation procedures, since polysome peaks were clearly visible in the A_{254} profiles of the fractionated gradients (e.g., Fig. 4A, fractions 6 to 10). To directly confirm the presence of polysomes associated with cellular mRNA, a portion of each gradient fraction was subjected to RT-PCR analysis using a primer

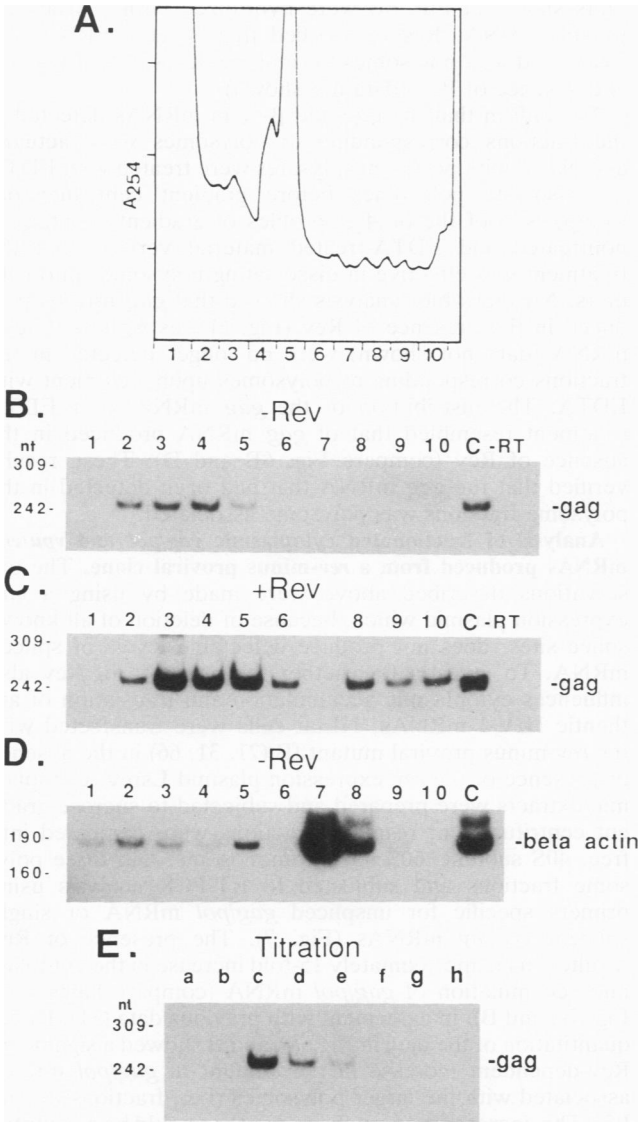

FIG. 4. RT-PCR analysis of fractionated cytoplasmic *gag* RNA. (A) Fractionation of cytoplasmic RNA. Cytoplasmic fractions of HLtat cells transfected with pCgagRRESpA with or without the *rev* expression plasmid pLsrev were subjected to centrifugation through sucrose gradients. Shown is a representative A_{254} profile indicating fractions. (B and C) RT-PCR analysis of fractionated *gag* mRNA. One-fifth of each gradient fraction was subjected to RT-PCR using primers specific for the *gag* gene. Resulting products were separated on nondenaturing 5% acrylamide gels. The expected size of the PCR-amplified product was 253 nt due to a 55-nt internal deletion which removed the major splice donor at nt 287 (25). Panels B and C show 15-min exposures of an analysis of the fractionated *gag* mRNA produced in the absence or presence of Rev, respectively. Lanes C contain amplification products prepared by using 0.5 μg of RNA isolated from the initial cytoplasmic extracts; −RT designates amplifications of cytoplasmic RNA preparations performed in the absence of reverse transcriptase. Positions of size markers and the 253-nt *gag* amplification product are indicated at the left and right, respectively. Fraction 7 of panel C was lost during the initial preparation. (D) RT-PCR analysis of fractionated mature β-actin mRNA. One-fifth of each gradient fraction was subjected to RT-PCR using primers specific for mature β-actin mRNA. Resulting products were separated on a nondenaturing 6% acrylamide gel. Shown is an analysis of the fractionated mature β-actin mRNA produced in the absence of Rev. The position of the expected 189-nt product is indicated at the right. (E) Titration of fractionated *gag* mRNA. Lanes: a, RT-PCR of 10 μl of fraction 9(−Rev) (see panel B); c to g, RT-PCR of serial fourfold dilutions of fraction 9(+Rev) (see panel C); a and h, no-reverse transcriptase controls of 10 μl of fractions 9(−Rev) and 9(+Rev), respectively.

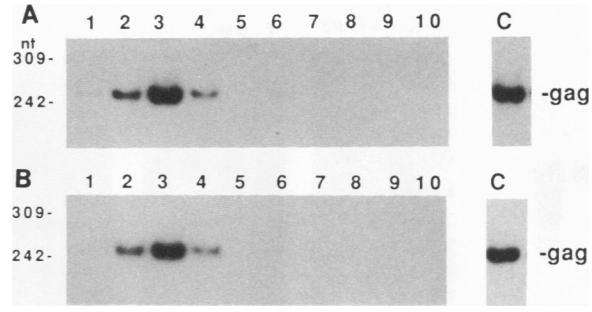

FIG. 5. RT-PCR analysis of fractionated cytoplasmic *gag* RNA produced in the absence of an RRE-Rev interaction. Cytoplasmic fractions of HLtat cells transfected with pCgagpA in the absence (A) or presence (B) of the *rev* expression plasmid pLsrev were subjected to sucrose gradient centrifugation followed by RT-PCR analysis using primers specific for the *gag* gene as described in Materials and Methods and the legend to Fig. 4. Lanes C contain amplification products prepared by using 0.13 μg of RNA isolated from the initial cytoplasmic extracts.

pair designed to amplify mature cytoplasmic β-actin mRNA. These results verified that β-actin mRNA was associated with polysomes both in the absence (Fig. 4D) and presence (not shown) of Rev. These observations demonstrated that the failure of *gag* mRNA to associate with polysomes in the absence of Rev was specific and did not result from disruption of polysomes during extraction or centrifugation. Results of an RNA titration experiment to assess the sensitivity of the RT-PCR technique demonstrated that a 16-fold mRNA dilution could easily be detected by this methodology (Fig. 4E). Therefore, the difference between the amount of mRNA associated with the polysomes in the presence or absence of Rev was substantially greater than could be accounted for by the fourfold increase in cytoplasmic *gag* RNA observed in this experiment. Taken together, these data demonstrated that the presence of Rev was required for efficient loading of *gag* mRNA onto polysomes.

The dependence of the interaction of Rev with the RRE on the cytoplasmic accumulation, ribosomal association, and translation of *gag* mRNA was studied by transfecting HLtat cells with pCgagpA in the presence or absence of a *rev* expression plasmid. This plasmid is identical to pCgagRRE SpA except that it does not contain the RRE. RT-PCR analysis of cytoplasmic RNA showed that, in contrast to results obtained with pCgagRRESpA, the presence of Rev did not increase the levels of cytoplasmic *gag* RNA produced from pCgagpA (Fig. 5, lanes C). Likewise, RT-PCR of sucrose gradient fractions revealed that the presence of Rev had no effect on the extent of ribosomal loading; i.e., the majority of the *gag* mRNA produced in both the absence and presence of Rev was detected in the gradient fractions corresponding to free and subunit-associated species (compare Fig. 5A and B). These observations were in general agreement with results of $p24^{gag}$ antigen capture assays which revealed no significant increase in the production of Gag protein by pCgagpA in the presence of Rev. Taken

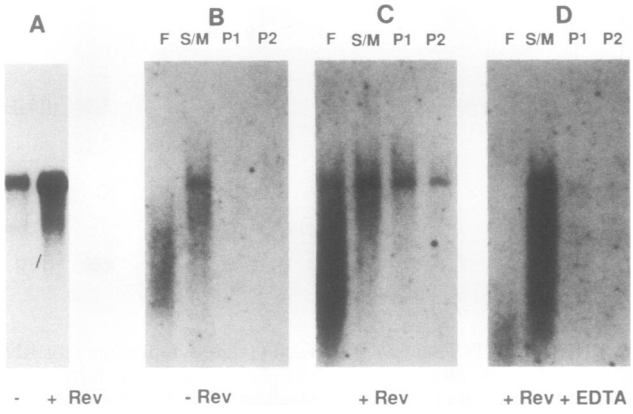

FIG. 6. Northern blot analysis of fractionated cytoplasmic *gag* mRNA. (A) Cytoplasmic RNA. Shown is a Northern blot of 20 μg of cytoplasmic RNA isolated from HLtat cells transfected with pCgag RRESpA in the absence (−) or presence (+) of the Rev expression plasmid pLsrev. The probe used to detect *gag* mRNA is indicated in Fig. 1. (B and C) Northern blot analysis of fractionated *gag* mRNA. The cytoplasmic extracts described for panel A were subjected to sucrose gradient centrifugation. Fractions as shown in Fig. 4A were collected and combined into free (F; corresponding to fraction 1), subunit/monosome (S/M; corresponding to fractions 2 to 5), and polysome (P1 and P2; corresponding to fractions 6 to 8 and fractions 9 and 10, respectively) pools. RNA was isolated and subjected to Northern blot analysis using the *gag* probe shown in Fig. 1. (D) Parallel analysis of a cytoplasmic extract that was incubated on ice with 5 mM EDTA for 10 min prior to centrifugation.

together, these results indicated that the increase in polysomal association and translation of *gag* mRNA was dependent on the presence of the RRE.

To verify that the RT-PCR method detected intact RNA, RNA fractionation was performed as in Fig. 4A, and RNA fractions were combined into free (corresponding to fraction 1 in Fig. 4A), subunit/monosome (corresponding to fractions 2 to 5) and polysome (P1 and P2; corresponding to fractions 6 to 8 and fractions 9 and 10, respectively) pools, subjected to Northern blot analysis, and hybridized to the *gag*-RRE probe shown in Fig. 1. The Northern blot shown in Fig. 6A demonstrated that the starting material that was applied to the gradients contained intact *gag* mRNA. Results of parallel analysis of the gradient fractions were in agreement with results obtained by using RT-PCR analysis (Fig. 6B and C). In the absence of Rev, *gag* mRNA was found in the free and subunit/monosome fractions; polysome-associated *gag* mRNA was detected only in the presence of Rev. In the absence of Rev, all of the *gag* mRNA in the free fraction was detected as degradation products; in the presence of Rev, this fraction contained both intact *gag* mRNA and degradation products. Interestingly, the majority of the *gag* mRNA detected in the subunit/monosome fractions in both the absence and presence of Rev was intact. Likewise, the polysome-associated *gag* mRNA detected in the presence of Rev was intact. The fact that the starting material was not significantly degraded (Fig. 6A) showed that degradation occurred during the centrifugation and fractionation procedures. The addition of inhibitors of RNA degradation (i.e., vanadyl ribonucleoside complexes, heparin, and RNasin) to lysates or gradient buffers did not significantly alter the extent of RNA degradation (data not shown).

To confirm that cellular mRNAs were associated with polysomes in the absence and presence of Rev, the Northern blots shown in Fig. 6B were hybridized with a probe for β-actin mRNA. Results verified that β-actin mRNA was associated with polysomes to similar extents in the presence and absence of Rev (data not shown).

To confirm that the *gag* and β-actin mRNAs detected in the fractions corresponding to polysomes were actually associated with polysomes, lysates were treated with EDTA to dissociate polysomes before gradient centrifugation. Comparison of the of A_{254} profiles of gradients containing nontreated and EDTA-treated material verified that the treatment was effective in dissociating polysomes into subunits. Northern blot analysis showed that *gag* mRNA produced in the presence of Rev (Fig. 6D) as well as β-actin mRNA (data not shown) were no longer detected in the fractions corresponding to polysomes upon treatment with EDTA. The distribution of the *gag* mRNA after EDTA treatment resembled that of *gag* mRNA produced in the absence of Rev (compare Fig. 6B and D). These results verified that the *gag* mRNA that had been detected in the polysome fractions was polysome associated.

Analysis of fractionated cytoplasmic *gag/pol* and *vpu/env* mRNAs produced from a *rev*-minus proviral clone. The observations described above were made by using a *gag* expression plasmid which, because of deletion of all known splice sites, does not produce detectable levels of spliced mRNA. To investigate whether the presence of Rev also influences cytoplasmic accumulation and translation of authentic HIV-1 mRNAs, HLtat cells were transfected with the *rev*-minus proviral mutant fB (21, 31, 66) in the absence or presence of the *rev* expression plasmid Lsrev. Cytoplasmic extracts were prepared and subjected to sucrose gradient centrifugation; resulting fractions were combined into free, 40S subunit, 60S subunit/monosome, and three polysome fractions and subjected to RT-PCR analysis using primers specific for unspliced *gag/pol* mRNA or singly spliced *vpu/env* mRNAs (Fig. 7). The presence of Rev resulted in an approximately 15-fold increase in the cytoplasmic accumulation of *gag/pol* mRNA (compare lanes C in Fig. 7A and B), in agreement with previous data (31). Direct quantitation of the data in Fig. 7A and B showed a significant Rev-dependent increase in the amount of *gag/pol* mRNA associated with the larger polysomes (i.e., fractions P2 and P3). This increase was much greater than could be accounted for by the increase in cytoplasmic *gag/pol* RNA (Table 1). These findings were in agreement with the observation that Gag protein production was increased by greater than 1,000-fold in the presence of Rev, as determined by p24 antigen capture assay. It is noteworthy that in the absence of Rev, a significant amount of *gag/pol* mRNA was detected in the first of the three polysome fractions (i.e., P1). This observation differs from data obtained by using the pCgagRRESpA *gag* mRNA (Fig. 4) and may reflect cross-contamination of adjacent fractions or a limitation in the resolution of different RNA-protein complexes imposed by studying the much larger authentic unspliced *gag/pol* mRNA. (It is estimated that the genomic-size *gag/pol* mRNA of approximately 10 kb will cosediment with monosomes even in the absence of any associated protein.) Figure 7C and D show that both the cytoplasmic accumulation and the polysomal association of singly spliced *vpu/env* mRNAs also were enhanced in the presence of Rev. Therefore, these results confirmed that the presence of Rev increased both cytoplasmic accumulation and polysomal association of RRE-containing HIV mRNAs. To confirm that these effects were specific for RRE-containing mRNAs, the levels and polysomal association of the multiply spliced *tat* mRNA, which does not contain the

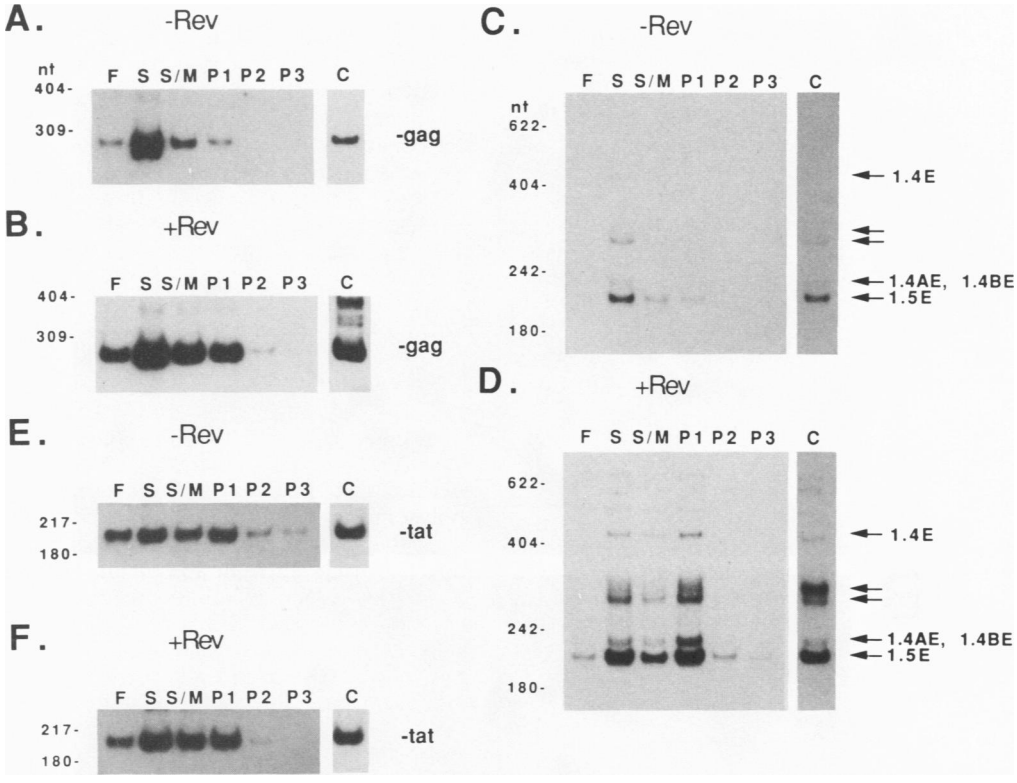

FIG. 7. RT-PCR analysis of fractionated cytoplasmic *gag/pol* and *vpu/env* mRNAs produced from a *rev*-minus proviral clone. Cytoplasmic extracts of HLtat cells transfected with the *rev*-minus proviral mutant fB (21, 31, 66) in the absence or presence of the *rev* expression plasmid pLsrev were subjected to sucrose gradient centrifugation. Resulting fractions were combined into free (F), 40S subunit (S), 60S subunit/monosome (S/M), and polysome (P1 to P3) fractions and subjected to RT-PCR analysis using primers specific for *gag/pol* mRNA (A and B), *vpu/env* mRNA (C and D), or *tat* mRNA (E and F) as described in Materials and Methods and the legend to Fig. 4. Lanes C contain amplification products prepared by using 0.26 μg of RNA isolated from the initial cytoplasmic extracts. Bands representing singly spliced *vpu/env* mRNAs are labeled according to their component exons; 1.4E includes the first exon of *tat* as well as the *vpu/env* open reading frames but expresses only the first exon of *tat* (63). Unlabeled arrows point to bands likely to represent *vpu/env* mRNAs containing the small noncoding exons 2 or 3 (63).

RRE, were determined by RT-PCR analysis of the same gradient fractions. As expected, the presence of Rev did not increase cytoplasmic accumulation of *tat* mRNA produced by the fB clone and did not increase the association of this mRNA with polysomes (Fig. 7E and F). Instead, Rev resulted in a slight decrease in the levels of the polysome-associated *tat* mRNA.

Localization of *gag* mRNA produced in the absence and presence of Rev by in situ hybridization. To localize *gag* mRNA within individual cells, in situ hybridizations were carried out on HLtat cells transfected with pCgagRRESpA, with or without a *rev* expression plasmid. The hybridizations were done by using a ^{3}H-labeled riboprobe complementary to *gag* mRNA (i.e., antisense); the corresponding sense riboprobe served as a control to detect a hybridization to plasmid DNA. Results demonstrated a clear difference in the localization of the *gag* mRNA (Fig. 8). In the presence of Rev, most of the grains representing *gag* mRNA were in the cytoplasm (Fig. 8C), while in the absence of Rev, most of the *gag* mRNA was in the nucleus and the region surrounding the nucleus (Fig. 8A and B). In agreement with results of biochemical fractionation experiments, a substantial proportion of cytoplasmic *gag* RNA was present in a subpopulation of cells (e.g., Fig. 8A). However, this cytoplasmic RNA did not show the same distribution as the cytoplasmic RNA produced in the presence of Rev. This finding suggested that the cytoplasmic *gag* RNA detected in biochemical fractionation experiments might not be in the same physical location in the presence and absence of Rev, consistent with the observed difference in the extent of polysomal loading. In control hybridizations, the antisense probe did not label cells transfected with carrier DNA alone, nor did the sense probe label cells transfected with pCgagRRESpA in the absence or presence of Rev (not shown).

TABLE 1. Quantitation of fractionated *gag/pol* mRNA produced from a *rev*-minus proviral clone[a]

Fraction	^{32}P counts		Fold induction
	−Rev	+Rev	
Free	14,940	147,736	10
40S subunit	356,937	711,450	2
Subunit/monosome	63,754	326,922	5
P1	16,322	266,343	16
P2	404	19,400	48
P3	0	11,469	>11,469

[a] The gels shown in Fig. 7A and B were scanned with an AMBIS radioanalytic imaging system. Presented are the total ^{32}P counts detected in each *gag* band, corrected for background.

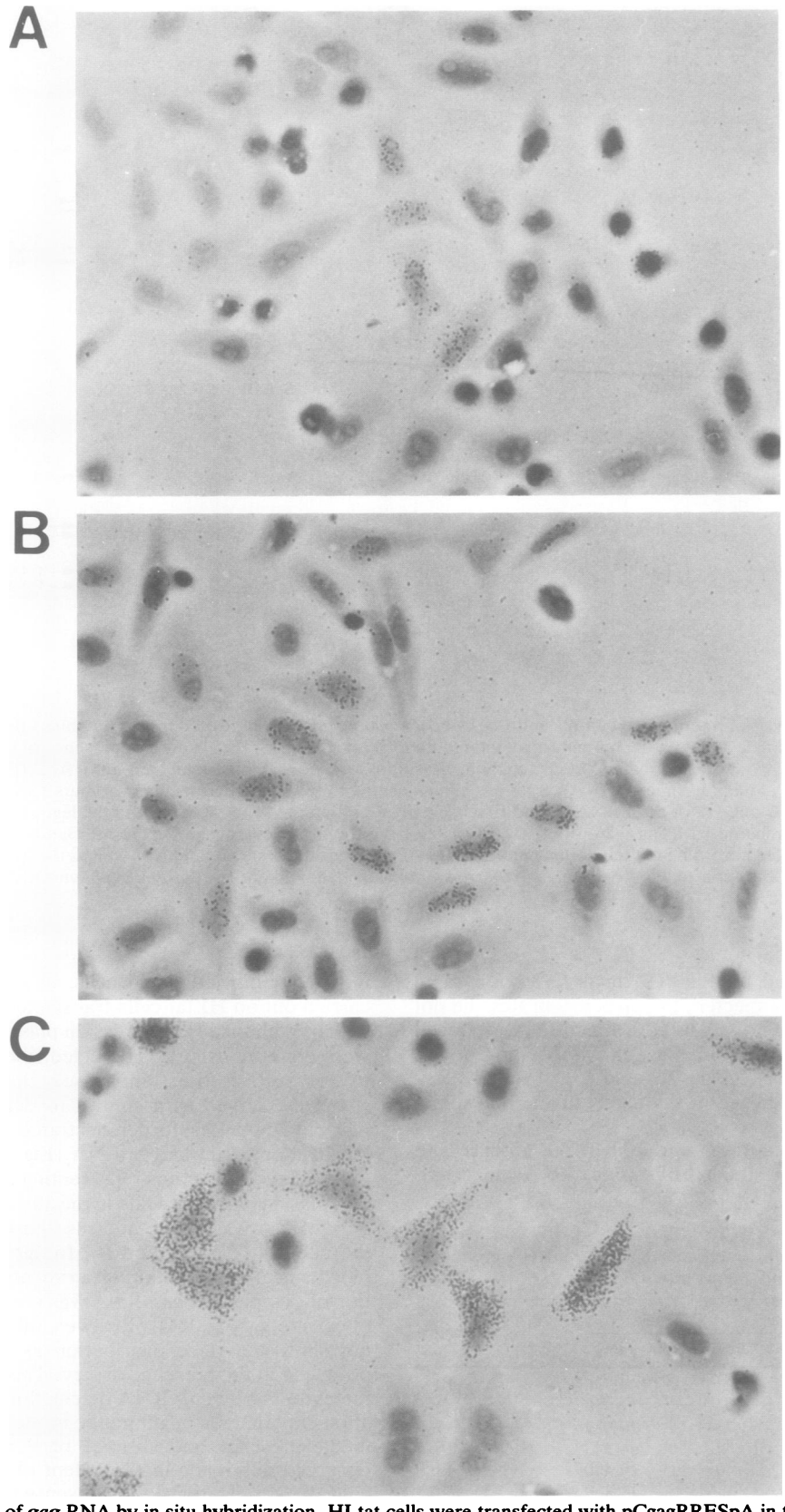

FIG. 8. Localization of *gag* RNA by in situ hybridization. HLtat cells were transfected with pCgagRRESpA in the absence (A and B) or presence (C) of the Rev-producing plasmid pLsrev and subjected to in situ hybridization using a ^{3}H-labeled RNA probe specific for *gag* RNA (see Fig. 1).

DISCUSSION

This report presents an analysis of the effect of Rev on the utilization of cytoplasmic RRE-containing mRNAs. By studying the subcellular localization of *gag* mRNA produced from a *gag* expression plasmid containing the RRE, we found that although *gag* mRNA was present in the cytoplasm in the absence of Rev, its translation into Gag protein was strictly dependent on the presence of Rev. Results of poly(A)$^+$ selection and in vitro translation experiments confirmed that the cytoplasmic *gag* mRNA produced in the absence of Rev was polyadenylated and functional. RNA fractionation experiments performed to determine the association of the *gag* mRNA with ribosomes revealed a significant difference in the extent of polysomal loading. The *gag* mRNA produced in the absence of Rev was almost completely excluded from polysome fractions. In contrast, a significant amount of the *gag* mRNA produced in the presence of Rev was found to be associated with polysomes. These results demonstrated that the presence of Rev regulates the translation of cytoplasmic *gag* mRNA at the level of polysomal loading. These findings were extended to the authentic unspliced *gag/pol* mRNA and to the group of singly spliced HIV-1 mRNAs that produce Env and Vpu proteins by studying the effect of Rev on the polysomal association of mRNAs produced by a *rev*-minus proviral clone. Results of in situ hybridizations showed that the cytoplasmic RNA detected in the absence of Rev was distributed differently than that produced in the presence of Rev. This finding was consistent with the observed difference in polysomal loading.

After the completion of this work, experiments by Arrigo and Chen (2) showed that in lymphoid cells the *env* mRNA is found in the cytoplasm in both the absence and the presence of Rev. The finding that the presence of Rev enhances the association of *gag/pol* mRNA and singly spliced *vpu/env* mRNAs with polysomes is in general agreement with results obtained by Arrigo and Chen (2), who concluded that Rev increases the translation of singly spliced mRNAs by promoting association with polysomes. It is noteworthy that while Rev increases the cytoplasmic accumulation of both unspliced and singly spliced mRNAs in a HeLa cell line (i.e., by approximately 15- to 20-fold; Fig. 7) and in COS cells (2), it has little or no effect on the cytoplasmic accumulation of singly spliced mRNAs in lymphoid cells (2). These observations may suggest that the presence of Rev exerts different effects depending on the cell type and specific mRNA. Alternatively, the definition of nuclear and cytoplasmic fractions by the biochemical separation methods applied by us and others may be an oversimplification; there may be more than one distinct pool in different physical locations.

As shown in Fig. 4, 6, and 7, the cytoplasmic *gag*-RRE mRNA produced in the absence of Rev migrated with the free, subunit, and monosome fractions. At present, we do not know whether the *gag* mRNA that sedimented in these fractions was actually bound to ribosomal subunits or whether it was associated with different particles. As shown in Fig. 6B to D, the mRNA found in the free fraction was extremely sensitive to degradation. This property may reflect the status of the *gag* mRNA found in the free fraction, especially in the absence of Rev. In this regard, it is noteworthy that earlier RNA fractionation experiments performed to characterize the regulation of ferritin mRNA also show the presence of degraded mRNA in the free fractions in the absence of translation (5). It is also interesting that even in the presence of Rev, a large proportion of the *gag* mRNA remained in the free, subunit, and monosome fractions, whereas β-actin mRNA was detected predominantly in the polysome fractions (compare Fig. 4C and D). This finding suggests that the *gag* mRNA produced in the presence of Rev is less readily loaded onto polysomes than are mRNAs encoding cellular proteins such as β-actin. One hypothesis explaining this difference is that HIV-1 mRNAs follow a transport pathway distinct from that of cellular mRNAs, which causes them to associate slowly with the translation machinery.

The functional activities attributed to Rev are thought to be mediated through direct binding of Rev to RRE RNA (6, 12, 14, 15, 34, 35, 43, 51, 71). Rev localizes primarily in the nucleoli of transfected cells (13, 25). Several experiments have indicated that Rev acts in the nucleus to promote the nucleus-to-cytoplasm transport of RRE-containing RNAs (20, 22, 25, 32, 45). The finding that Rev also plays a regulatory role at the level of translation raises the question as to whether Rev remains associated with the mRNA in the cytoplasm; this question is currently under investigation. Biochemical fractionation experiments have demonstrated the presence of substantial quantities of Rev in the cytoplasm (21a).

Although regulation of gene expression at the transcriptional and translational levels has been studied in detail, regulatory steps occurring between these stages are not well understood. To date, only a few mRNA-binding factors important for RNA regulation have been identified and characterized. These include Rev and its human T-cell leukemia virus type 1 (HTLV-1) homolog, Rex (1, 19, 23, 33, 36, 37, 39, 56, 65, 70), which are responsible for regulating the expression of viral proteins encoded in unspliced and partially spliced viral mRNAs, and the cellular protein IREBP (iron-responsive element [IRE]-binding protein) (for a review, see reference 40). This protein coordinately regulates both a decrease in expression of ferritin mRNA at the level of protein translation and an increase in expression of transferrin receptor (TfR) mRNA at the level of mRNA stabilization.

Comparison of Rev and Rex with IREBP reveals interesting similarities and differences between the two systems. Rev, Rex, and IREBP each interact with *cis*-acting RNA elements named RRE, RXRE, and IRE, respectively, which contain stem-loop structures. IREBP recognizes both an IRE located in the 5′ untranslated region of ferritin mRNA and a group of IREs located 3′ to the coding region of the TfR mRNA. Interaction with $IRE_{ferritin}$ leads to suppression of polysomal loading, while interaction with IRE_{TfR} stabilizes the mRNA. The interaction of Rev with RRE increases the stability, transport, and polysomal loading of mRNA. Therefore, the Rev-RRE and IREBP-IRE_{TfR} interactions are similar in that both have positive effects on the mRNA. Both the Rev-RRE and IREBP-$IRE_{ferritin}$ interactions affect RNA expression at the level of translation, but with opposite effects. Although HTLV-1 Rex is known to promote the transport of unspliced and singly spliced viral mRNAs to the cytoplasm (23, 33), it is not yet known whether Rex influences mRNA stability and polysomal loading. An additional similarity between the TfR and RRE-containing mRNAs is the presence of elements that destabilize the RNA. In the case of TfR mRNA, a destabilizing element has been identified within the domain containing the IREs, the effect of which is counteracted by the IREBP-IRE interaction (8). Likewise, in addition to RRE, the HIV-1 mRNAs encoding Gag, Pol, and Env have been proposed to contain sequences which inhibit their expression. These sequences are different

from, and act independently of, RRE. Several inhibitory sequences have been identified in the *gag* gene (64) and *env* gene (48, 58). One of these sequences, termed INS-1, has been shown to lower the steady-state levels of *gag* mRNA (64). The instability effect caused by INS-1 is overcome in the presence of the Rev-RRE interaction. Other postulated sequences within *env*, named CRS, may influence the stability, transport, or utilization of the viral mRNAs (58), but their exact role has not been determined.

Our findings provide direct evidence that the presence of Rev is required along the entire pathway of mRNA transport and utilization to enable the stabilization, correct localization, and efficient translation of RRE-containing viral mRNAs. This also highlights a difference between Rev and IREBP: while IREBP exerts its effects on IRE-containing mRNAs in the cytoplasm, Rev influences the fate of RRE-containing mRNAs both in the nucleus and after they reach the cytoplasm.

Two general models have been proposed to explain the mechanism of Rev function. Chang and Sharp proposed that Rev acts by dissociating spliceosome components from pre-mRNAs, an effect requiring the presence of suboptimal or slow splice sites (10). In a second model, Rev is proposed to direct RRE-containing mRNAs through an alternative transport pathway, possibly involving the nucleolus (25, 52). The finding that the presence of Rev influences the translation of viral mRNAs adds to the complexity of the Rev phenotype. This effect cannot be accounted for by dissociation of RRE-containing mRNAs from the splicing apparatus and provides evidence in support of an extension of the second hypothesis stated above. In this model, Rev acts as a chaperone to guide RRE-containing mRNAs through a specific pathway which is initiated in the nucleus immediately after transcription and leads to translation. While it is clear that this pathway must provide partial protection from splicing and degradation within the nucleus, we do not yet fully understand the events that take place in the cytoplasm, which ultimately lead to increased polysomal loading and translation. Although our data provide strong evidence for an effect at the level of polysomal loading, at present we cannot rule out the possibility that Rev affects translation via more than one mechanism. One possibility is that the presence of Rev results in the dissociation of a cellular protein which binds to the RRE-containing mRNA and prevents it from being translated. Another possibility is that the presence of Rev dissociates the RRE-containing mRNAs from an as yet uncharacterized ribonucleoprotein particle and promotes their loading on the polysomes. Alternatively, the presence of Rev might influence the location of the viral mRNA after it reaches the cytoplasm. It is anticipated that further elucidation of this complex pathway of regulation of viral mRNAs will aid a more clear understanding of the pathways important for the cellular regulation of mRNA sequestering, stability, and translation.

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