rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA

(posttranscriptional regulation/splicing/acquired immunodeficiency syndrome)

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ABSTRACT rev (trs/art) is an essential human immunodeficiency virus type 1 (HIV-1) regulatory protein. rev increases the levels of the gag- and env-producing mRNAs via a cis-acting element in the env region of HIV-1, named rev-responsive element. Our results show that rev increases the stability of the unspliced viral mRNA, while it does not affect the stability of the multiply spliced viral mRNAs that do not contain the rev-responsive element. The study of mutated proviral constructs producing mRNA that cannot be spliced revealed that the effect of rev on stability is independent of splicing. Our experiments also indicate that rev promotes the transport of the viral mRNA containing the rev-responsive element from the nucleus to the cytoplasm. The proposed functions of rev are consistent with its nuclear localization as shown by immunofluorescence. The selective effects of rev on the levels of the viral mRNA suggest a model for feedback regulation by rev leading to a steady state of viral expression.

Two known human immunodeficiency virus type 1 (HIV-1) proteins, tat and rev (formerly known as art or trs), expressed from partially overlapping reading frames, are essential for the expression of HIV-1 in human cells. The transactivator or tat protein (1–5) has been shown to increase the steady-state levels of all HIV-1 mRNAs as well as of mRNAs produced by recombinant constructs containing the HIV-1 LTR promoter and R region ligated to a variety of indicator genes (6–12). rev is required for efficient production of the HIV-1 structural proteins (13–16). Different hypotheses for the function of rev have been proposed. Sodroski et al. (13) proposed that rev acts posttranscriptionally as an "anti-repressor" and that it does not affect the levels of viral mRNAs. Feinberg et al. (14) reported that rev-defective mutants displayed an altered splicing pattern and did not produce any genomic-length RNA. Knight et al. (15) also reported that both tat and rev were necessary for env protein production from a recombinant construct.

HIV-1 produces three size classes of mRNA: unspliced, singly spliced, and multiply spliced. We have shown that rev mutants produce low levels of unspliced and singly spliced mRNA (16), resulting in low levels of gag and env protein expression. In addition, no infectious virus is produced by these mutants. The presence of the rev protein in trans fully complements all of the defects of the rev mutants. Our studies suggested the presence of two different cis-acting elements responsible for the low levels of mRNA within the gag region (localized between nucleotides 287 and 1543) and the env region (localized between nucleotides 5923 and 7955). These elements are not responsive to the presence of rev. A third cis-acting element, named rev-responsive element (RRE) and also localized within the env region of HIV-1, is required in cis for rev function. RRE is responsible, in the presence of rev, for the increased steady levels of unspliced (gag-producing) and singly spliced (env-producing) mRNA (16). Rosen et al. (17) have also reported that an element in the env region of HIV-1 was responsible for increased chloramphenicol acetyltransferase (CAT) production from hybrid HIV-CAT gene constructs.

In this report, we analyze the mechanism of rev function. We demonstrate that rev increases the half-life of the unspliced viral mRNA, while it does not affect the half-life of the multiply spliced viral mRNAs that do not contain the RRE. The study of mutated viral mRNAs produced by recombinant constructs reveals that rev increases the stability of the mRNAs independent of splicing. In addition to the stabilization of the RRE-containing mRNAs, our experiments suggest that rev decreases the amount of mRNA that is targeted for splicing, and it also may affect the transport of the unspliced viral mRNA from the nucleus to the cytoplasm.

MATERIALS AND METHODS

Plasmid Constructs. The proviral deletion mutants have been described (16) or are described in Fig. 1.

Transfections. Plasmid DNA was transfected into HeLa cells by the calcium phosphate coprecipitation technique (24) as described (19), except that the cells were not treated with glycerol. Next day the medium was changed twice within 1 hr to wash out the remaining precipitate. On the second day, the cells were analyzed for RNA or protein production.

RNA Analysis. RNA was isolated by the hot phenol (20) or heparin–DNase I (21) method. The cells were rinsed with ice-cold phosphate-buffered saline (PBS), scraped from the plates in PBS, and pelleted at 1000 rpm in a Sorvall RT 6000 centrifuge. Nuclei were prepared from detergent-treated cells (22) by homogenizing with a Dounce homogenizer and pelleting at 1500 rpm. Cytoplasmic RNA was prepared from the supernatant as described above. The nuclei were resuspended in 1 ml of lysis buffer without detergent and purified by sedimentation through 10 ml of 30% sucrose in lysis buffer (1500 rpm for 10 min). Nuclear RNA was prepared as described above. Because of the purification protocol, the nuclear RNA fraction contains substantial amounts of cytoplasmic RNA, while the cytoplasmic fraction has little contamination with nuclear RNA. The RNA was analyzed by blot hybridization (Northern blot) and S1 nuclease protection techniques. For the Northern blots, the RNA was detected by hybridization to a nick-translated Xho I–HindIII fragment (nucleotides 8473–9192) of HXB2c as described (16). This probe hybridizes with both the 5′ and 3′ ends of the viral mRNAs. The S1 nuclease probe spanning the splice donor at position 287 has been described (16). The amounts of spliced and unspliced mRNA were determined by liquid scintillation

Abbreviations: HIV-1, human immunodeficiency virus type 1; RRE, rev-responsive element; LTR, long terminal repeat.

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The BssHII-Cla I fragment of pMVgag (nucleotides 254–373) was replaced by a synthetic oligonucleotide, resulting in a deletion of nucleotides 260–326. To construct plasmids pMVgagA2 and pCgagA2, a Bgl II–HindIII fragment (A2, nucleotides 7197–7717) of HXB2c was inserted into the Xho I site of pMVgag and pCgag, respectively. To construct pCgagA1 (not shown), a Bgl II–BamHI fragment (A1, nucleotides 7197–8051) was inserted into the Xho I site of pCag. Fragments A1 and A2 contain the cis-acting RRE (16). (E) rev-producing plasmid pL3ctrs. To generate pL3ctrs, the intronless rev sequence (nucleotides 5545–8473) from pMctrs (16) was inserted into an intermediate vector, pL3pa, which contains the HIV-1 LTR promoter (nucleotides −1064 to +80) and a Bcl I–BamHI fragment of simian virus 40 DNA containing the polyadenylylation signal. pL3ctrs does not contain the tat initiator AUG. Therefore, the first AUG at position 5549 is the rev initiator AUG. pA, polyadenylylation signal; A, Apa I; Bc, Bcl I; B, BamHI; C, Cla I; E, EcoRI; H, HindIII; S, Sal I; X, Xho I.

RESULTS

Subcellular Localization of mRNAs. To study the effects of rev on the viral mRNA, the proviral mutants were transfected into human HeLa cells, and their expression was studied at the RNA and protein level in the presence or absence of rev protein.

The subcellular localization of the different species of viral mRNAs was determined by separating nuclei and cytoplasm of transfected cells and isolating RNA from these fractions. The percentage of unspliced mRNA in the two fractions was determined by S1 nuclease protection analysis with a probe that spans the first splice donor located at position 287 and can distinguish the unspliced from the spliced mRNA species. The fractions of spliced and unspliced mRNA were quantitated by liquid scintillation counting as described (16).

The percentage of unspliced mRNA was 70% in the nuclear and 60% in the cytoplasmic fractions of cells transfected with the intact proviral clone HXB2c (Fig. 2, lane 1). Similar levels of both unspliced and spliced mRNAs exist also in the nucleus of cells transfected by the rev− mutants fB and ΔX11 (lanes 2 and 4, respectively). However, the amount of cytoplasmic unspliced mRNA produced by mutants fB (lane 2) and ΔX11 (lane 4) was greatly decreased when compared with the amount produced by HXB2c. The percentage of unspliced cytoplasmic mRNA of fB and ΔX11 was 13% and 26%, respectively. The presence of rev in trans increased the percentage of the unspliced mRNA in the cytoplasm to ~50% for both mutants (lanes 3 and 5). The nuclear levels of both unspliced and spliced mRNA were not affected substantially by rev. The levels of RNA produced by HXAgag were similar to the RNA levels produced by HXB2c (lane 6, see below).
These results indicated that similar amounts of unspliced mRNAs exist in the nucleus, independent of the presence of rev. The dramatic decrease in the percentage of unspliced mRNA in the cytoplasmic fraction in the absence of rev could be caused by the decreased stability of the unspliced mRNA in the cytoplasm. Alternatively, changes in the splicing rates in and the transport of the viral mRNA could also contribute to this decrease.

**rev Affects the Stability of the Unspliced mRNA.** To measure the stability of the various mRNAs in the presence or absence of rev, transcription was blocked by addition of actinomycin D in cells transfected with various proviral mutants. The level of total unspliced mRNA was quantitated for up to 6 hr after addition of actinomycin D by using the S1 nuclease assay. All viral mRNAs are transcribed from the same promoter; therefore, a change in the relative levels of unspliced to spliced mRNA must reflect changes in relative stability and/or rate of splicing.

Both spliced and unspliced mRNAs produced by HXB2c were stable in the presence of actinomycin D (Fig. 3 Left, HXB2). The amount of unspliced mRNA produced by the rev− mutant fB decreased rapidly within the first hour after actinomycin D addition (Fig. 3 Left, fB). Therefore, the stability of the unspliced mRNA was lower in the absence of rev. The estimated half-life of the unspliced mRNA was less than 1 hr. The stability of the spliced mRNA was not significantly affected by rev, indicating that rev does not directly affect the spliced mRNA. The levels and stability of mRNA produced by fB in the presence of rev in trans (Fig. 3 Left, fB+pMctrs) were similar to those produced by HXB2c. Similar results were obtained when using either total or cytoplasmic mRNA (data not shown). The significance of the higher level of spliced mRNA produced by fB in the absence of rev is discussed below (see Discussion).

**The RRE Sequence Is Responsible for the mRNA Stabilization.** We have shown that the presence of a 520-base-pair fragment containing the RRE is responsible for efficient gag mRNA and protein production from vector pMVgagA2 (Fig. 1; see ref. 16). This plasmid contains only one splice donor of HIV-1 at position 287. After transfections into HeLa cells, a spliced mRNA was produced in addition to unspliced mRNA by using the HIV-1 splice donor and a cryptic splice acceptor (16). In the absence of rev protein, this spliced mRNA was the most prominent, while in the presence of rev, the amount of the unspliced mRNA increased at the expense of the spliced species. We measured the stability of these mRNAs using actinomycin D (Fig. 3 Right). The half-life of the unspliced mRNA was <1 hr in the absence of rev and was increased to >4 hr in the presence of rev (Fig. 3 Right, MVgagA2+pMctrs). The spliced mRNA did not show any change in stability. Therefore, the results of these experiments were similar to those obtained with mutant fB (Fig. 3 Left), indicating that, despite the extensive deletions in pMVgagA2, the viral mRNAs produced were regulated in a similar manner. These results also showed that RRE is responsible for the stabilization of the unspliced mRNA in the presence of rev.

**Stabilization of RRE-Containing mRNA Is Not a Result of Envelopment.** The unspliced mRNAs of retroviruses, in addition to being the messages for gag and gag-pol polyproteins, also serves as genomic RNA that is encapsidated into the virion. Transfections of HeLa cells by HXB2c resulted in the production of infectious virus; therefore, some of the unspliced mRNA was encapsidated into the virions. Neither fB nor ΔX11 can produce virus in the absence of rev (16), and their unspliced genomic mRNA is therefore not encapsidated. In the presence of rev, viral particles are formed (16) that encapsidate unspliced viral mRNA. This could result in increased stability of these RNAs. To test this hypothesis, we constructed a rev− viral mutant, HXΔgag, which has an extensive deletion in the gag region of the virus (see Fig. 1) and cannot produce viral particles. HXΔgag was transfected into HeLa cells, and the nucleolar and cytoplasmic mRNAs produced were compared by S1 nuclease protection analysis (Fig. 2, lane 6). The amount of unspliced mRNA produced by HXΔgag was similar to that of HXB2c (lane 1), indicating that the differences in the stability of unspliced mRNA observed between rev− constructs (such as fB and ΔX11) and HXB2c were not...
were not the result of encapsidation of the unspliced viral mRNAs.

The Effect of rev on Stability Is Independent of Splicing. To distinguish between the effects of rev on stability and on splicing, we constructed proviral mutants that respond to rev and do not contain any functional splice sites. These mutants are derivatives of pMVgag (16) (see Fig. 1), which contains only one known HIV-1 splice donor at position 287 and produces spliced mRNA (see Fig. 4 Left and Center). To eliminate the possibility of splicing, the splice donor at position 287 was deleted from pMVgag and pMVgagA2, producing plasmids pCgag and pCgagA2 (see Fig. 1). By using Northern blot analysis (Fig. 4 Left), it was verified that these constructs did not produce any detectable spliced mRNA. Low levels of unspliced mRNA and of gag protein were produced by pCgag and pCgagA2 in the absence of rev protein (Fig. 4 Left and Right, respectively). The presence of rev caused the accumulation of more full-length mRNA and gag protein from pCgagA2 but not from pCgag. This experiment suggested that rev affected the stability of RRE-containing mRNA in the absence of splicing.

rev Is Localized in the Nucleus. To study the subcellular localization of rev protein, plasmid pL3cts (transfected into HeLa cells together with the tat-producing plasmid pMX3N (6). Analysis by the indirect immunofluorescence technique using a specific antibody raised against the C-terminal part of rev revealed that the rev protein was localized in the nucleus of the transfected cells (Fig. 5A). Phase microscopy of the same field (Fig. 5B) showed that the strongly fluorescent areas in the nucleus corresponded to nucleoli. Similar results were obtained after transfection of the plasmid pMCts (16) into COS cells (data not shown). Control experiments demonstrated that nontransfected and mock-transfected cells did not react with the antibody.

DISCUSSION

The levels of gag and env mRNAs produced by HIV-1 in the absence of rev protein are low (14–16). Our results suggested that two independent regions within gag and env are responsible for the low levels of gag and env mRNA in the absence of rev (16). The presence of the RRE in cis and of the rev protein in trans are necessary for the accumulation of these mRNAs.

Stability measurements in the presence of actinomycin D strongly suggest that the rev protein increases the stability of the unspliced viral mRNA. The stability of the multiply spliced mRNAs is not significantly affected by rev. These multiply spliced mRNAs, unlike the unspliced viral mRNA, are stable and do not require rev.

If rev only affected the stability of the unspliced mRNA, we would expect the same amount of spliced mRNA in both the absence and presence of rev. Since the levels of multiply spliced mRNA decrease in the presence of rev, other functions, in addition to mRNA stabilization must be attributed to rev. These functions could include effects on the transport of viral mRNAs and changes in the splicing rates. To evaluate the latter possibility, we constructed viral deletion mutants that depend on the presence of rev protein and do not contain any functional splice sites. The study of pCgag, pCgagA1, and pCgagA2, which do not produce any spliced mRNA, revealed that the effects of rev are independent of splicing. The study of the HIV-1 env-expressing plasmid pL3ten, which does not release any spliced mRNA, also supports this conclusion (16).

The difference in levels of unspliced mRNA in the nuclear and cytoplasmic fractions suggests that, in the absence of rev, the transport of unspliced mRNA from the nucleus to the cytoplasm is impaired by rev.
cytoplasm is defective. In such a case, one might expect accumulation of unspliced mRNA in the nucleus, which was not observed. The lack of accumulation of large quantities of unspliced mRNA of the rev\textsuperscript{-} mutants in the nucleus might reflect the fact that this mRNA is channeled towards a splicing pathway. The spliced mRNA is stable and could be efficiently transported to the cytoplasm for translation. This hypothesis would explain why, in the absence of rev, more spliced mRNA is produced (see Fig. 3). If the mRNA cannot be spliced (for example pCgag and pCgagA2), it may be degraded in the nucleus, resulting in low steady-state levels. Therefore, the rev\textsuperscript{-} phenotype could be the result of defects in transport as well as mRNA stability.

A model summarizing the proposed activities of rev protein is shown in Fig. 6. rev, or a cellular factor induced by rev, interacts with the cis-acting element (RRE) in the env region of HIV-1. This channels the unspliced mRNA towards transport and away from splicing. In addition, this interaction stabilizes the unspliced mRNA.

rev appears to decrease the amount of mRNA that is available for splicing; therefore, it should act in the nucleus prior to the splicing of the nascent transcripts. The localization of the rev protein in the nucleus and especially in the nucleoli of transfected cells is consistent with the proposed function of rev. However, our experiments cannot exclude the possibility that the concentration of rev in the nucleoli of transfected cells may be an artifact of overexpression. If rev is an RNA binding protein, it could be attracted to the nucleoli, which contain large quantities of ribosomal RNA. It should be mentioned that low levels of rev that are undetectable by our immunofluorescence or immunoprecipitation assays can also complement the rev-defective mutants.

The opposite effects of rev on the levels of spliced and unspliced mRNA suggested a model for feed-back regulation of viral expression. We have proposed that rev negatively regulates the levels of tat protein expressed after viral infection and is responsible for the establishment of a steady state for the balanced expression of all viral proteins (†, 25). We have been able to confirm this model by direct measurements of the levels of tat and rev proteins and of the corresponding mRNAs (unpublished data).

The specific HIV-1 regulatory factors constitute promising targets for novel antiviral strategies. The elucidation of the mechanism of their function will certainly aid the development of appropriate strategies.

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