Rev-dependency of expression of human immunodeficiency virus type 1 *gag* and *env* genes

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Abstract Structural gene expression of human immunodeficiency virus type 1 (HIV-1) requires a viral regulatory protein, Rev transactivator. We investigated Rev-dependency of HIV-1 gene expression by various reporter systems. Expression of unspliced and singly-spliced viral mRNAs was demonstrated to be differentially dependent on the Rev function. This difference of Rev-dependency was found not to be determined by *cis*-elements in *gag*, *pol*, and *env* coding sequences reported so far, and was lost when the reporter constructs containing minimum elements for Rev-responsiveness such as splice signals and rev responsive element were used for experiments. These findings indicated that fundamental structure of HIV-1 mRNA was critical for the differential regulation of gene expression by Rev transactivator.

Key words: HIV-1; Rev-dependency; *gag*; *env*; *nef*

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is categorized under *lentivirus* by its complicated genome structure. *Lentivirus* is distinct from *oncovirus* such as murine and avian leukemia viruses in its complex mechanism of gene expression [1–3]. Among various regulatory genes, *rev* gene is commonly found in almost all lentiviruses [4–11] and function of HIV-1 Rev has been extensively studied [12–14]. Rev protein of HIV-1 positively modulates virion production through up-regulation of mRNA transport into cytoplasm by interaction with its RNA target, Rev responsive element (RRE) [13,14]. It has been postulated that inhibitory sequences named *cis*-acting repression sequence (CRS) [14,15], inhibitory sequence (INS) [16,17] and *cis*-acting inhibitory region (IR) [18] in coding regions of structural genes prevent transport of mRNAs for these genes into cytoplasm, and Rev protein relieve this supression. The other assumption of Rev function is that splice signals present in HIV-1 genome confer Rev-dependency on some species of HIV-1 mRNAs [13,19–21].

HIV-1 mRNAs are classified into three major groups by their size [22]. Full-length mRNA serves as viral genomic RNA and encodes *gag* and *pol* genes. Medium-size mRNAs (singly spliced) encode *vif*, *vpr*, *vpu*, and *env* genes. Small-size mRNAs (multiply spliced) encode *tat*, *rev*, and *nef* genes. It has been reported that *rev* gene function was required for expression of full- and medium-length mRNAs, by which virus structural proteins were encoded [23,24]. In this report, we examined Rev-requirement of the three groups of mRNAs by various assay systems and found that expression of full- and medium-length mRNAs was differentially dependent on Rev function. Deletion analysis revealed that most of the *cis*-acting inhibitory sequences reported previously [14–18] did not contribute either to this difference and to Rev functionality. Our data strongly suggested that fundamental elements on mRNAs such as splice signals had crucial role for regulation of gene expression by Rev transactivator.

2. Materials and methods

2.1. Cell lines and DNA transfection

A human colon carcinoma cell line, SW480 [25], a monkey kidney cell line, Cos-7 (ATCC CRL 1651), a hamster kidney cell line, BHK21 (ATCC CCL 10), a murine cell line, SC-1 [26], and a feline kidney cell line, CRFK (ATCC CCL 94), were maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum. A CD4-positive human T-cell leukemic cell line, A3.01 [25], and a CD4-positive human histiocytic lymphoma cell line, U937 (ATCC CRL 1593), were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum. For transfection of adherent (SW480, Cos-7, BHK21, SC-1, CRFK) and non-adherent (A3.01, U937) cells, calcium phosphate coprecipitation [27] and modified DEAE-dextran [28] methods were used, respectively.

2.2. CAT assay

The chloramphenicol acetyltransferase (CAT) assay has been previously described [29]. CAT levels were assayed in equivalent amounts of cell lysates prepared from transfected cell lines. For quantitation, radioactivity of spots corresponding to acetylated and non-acetylated forms of chloramphenicol on thin layer chromatograms was determined by bio-imaging analyzer BAS2000 (Fuji Photo Film, Corp., Tokyo, Japan). Transfection with a plasmid pSVβGAL (Promega Co. Ltd, WI, USA) producing β-galactosidase was used as a control for transfection efficiency. Comparable levels of bGAL were produced in each experiment.

2.3. RT assay

Virion-associated reverse transcriptase (RT) activity was measured as described previously [30]. For quantitation, radioactivity of spots on DE81 paper (Whatmann International Ltd., Maidstone, England) was determined by bioimaging analyzer BAS2000.

2.4. Preparation of cytoplasmic RNA and Northern blotting analysis

Cytoplasmic RNA was prepared from transfected SW480 cells by NP40-disruption [31] and AGPC [32] methods. An equal amount of cytoplasmic RNA (5 μg) was electrophoresed through 1.2% agarose gels containing 2.2 M formamide, and analyzed by Northern blot hybridization [31]. As a DNA probe for Northern hybridization, a cut-out DNA fragment (about 0.5 kb long) containing virus long terminal repeat was labeled with ^{32}P by the random primer DNA labeling kit (Takara shuzo, Co., Ltd., Kyoto, Japan).

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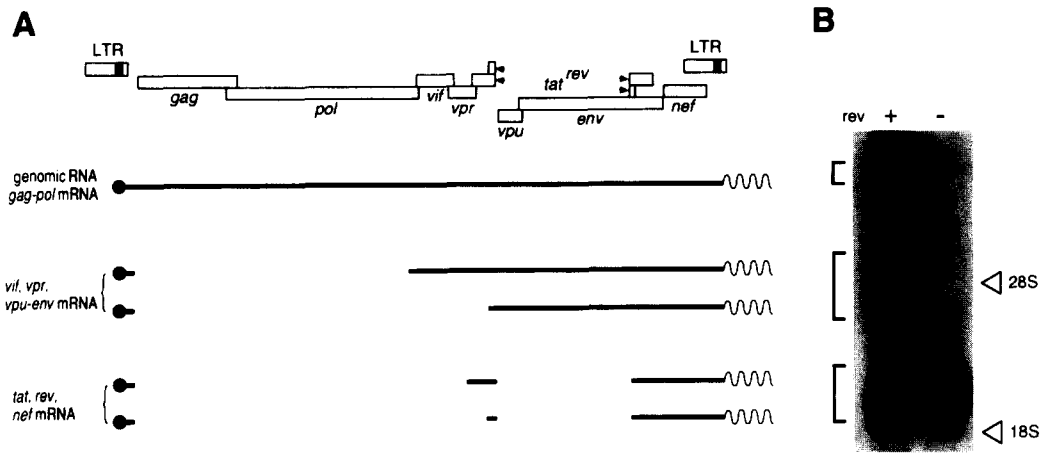

Fig. 1. Expression of cytoplasmic mRNAs regulated by Rev transactivator. (A) Major three species of HIV-1 mRNAs and the genes coded for by these mRNAs. Medium- and short-length mRNAs contain many hetelorogous species of mRNAs [22], but only major species are presented. (B) Cytoplasmic mRNAs of HIV-1 analyzed by Northern blot analysis. A Rev-defective proviral DNA clone, designated pNL-Ba [8] (10 μg), was transfected into SW480 cells with a Rev-expression plasmid (+), prevl [21] (20 μg), or with its backbone plasmid (−), pRVSV [21] (20 μg) and 2 days later, cytoplasmic RNA was prepared. An equal amount of RNA (5 μg) was used for Northern blot hybridization. The positions of three species of HIV-1 mRNAs and of rRNAs (28S and 18S) are indicated by brackets and arrowheads, respectively.

2.5. DNA construction

All CAT containing viral reporters are derived from an infectious DNA clone of HIV-1 designated pNL432 (GenBank Accession No. M19921)[25]. pNLgCAT is previously referred to as pNLgCAT-R1 [8]. pNLeCAT was constructed by insertion of a CAT-containing fragment (*Hin*dIII–*Sau*3AI fragment of pSV2cat) [29] into *Ssp*I(nt 6,153)–*Dra*III(nt 6,591) sites of pNL432 (Fig. 2). pNLgCATΔ was constructed by a deletion of *Spe*I–*Bal*I fragment(nt 1,507 to 4,551 of pNL432) from pNLgCAT. pNLeCATΔ was made by a deletion of *Dra*III–*Ssp*I fragment(nt 6,591 to 7,556 of pNL432) from pNLeCAT. These reporters all have a frame-shift mutation in *rev* gene, as was described for a Rev mutant proviral clone pNL-Ba [8]. Another reporter clone pNLnCAT was previously described [33]. pRSp-gCAT was constructed by cutting out *Bss*HII–*Pfl*MI fragment (nt 711 to 5,297 of pNL432) from pNLgCATΔ and inserting this into an expression vector pRVSV [21] (Fig. 2). pRSp-eCAT is previously referred to as pRSpCAT-R1 [21]. A Rev expression plasmid, prevl, was described previously [21].

3. Results

3.1. Rev-dependency of expression of HIV-1 mRNAs

Three major species of HIV-1 mRNAs were readily recognized by Northern blotting analysis. To investigate dependency of their expression on Rev regulatory protein, we examined Rev-requirement for cytoplasmic accumulation of each species of viral mRNAs. A Rev mutant proviral DNA clone, designated pNL-Ba [8], was transfected into SW480 cells in the presence or absence of a Rev expression plasmid, and expression of HIV-1 mRNAs was monitored. As shown in Fig. 1, full-and medium-length mRNAs depended upon Rev co-expression for their accumulation in cytoplasm. However, the medium-size mRNAs appeared to be expressed to a small extent in the absence of Rev. Table 1 shows quantitative data of the Northern analysis. Although the amount of the full-length mRNA in cytoplasm was almost negligible without Rev protein, medium-length mRNAs were definitely present in cytoplasm in the same condition. Enhancement of accumulation of medium-length mRNAs by Rev transactivator was low relative to that of the full-length mRNA (Table 1). In contrast to these two mRNA species, expression of short-length mRNAs was essentially independent on Rev protein (Fig. 1 and Table 1). These results showed that expression of each HIV-1 mRNA species was differentially regulated by Rev transactivator.

3.2. Differential regulation of HIV-1 gene expression by Rev transactivator in various cell lines

Three reporter clones, designated pNLgCAT, pNLeCAT and pNLnCAT, were constructed to determine responsiveness of each mRNA species to Rev protein more easily and sensitively (Fig. 2). CAT activity produced in the cells transfected with pNLgCAT, pNLeCAT or pNLnCAT was expected to represent expression of full-, medium- and short-length mRNAs in cytoplasmic fraction, respectively. To ascertain whether these reporters show Rev-responsiveness similar to that of three mRNA species, each of reporters was transfected into SW480 cells with or without a Rev expression plasmid and enhancement of CAT activity by Rev was determined (Table 2). The results obtained with these 3 reporters were almost parallel with those of cytoplasmic mRNA analysis in Fig. 1 and Table 1. Drastic enhancement of CAT production by Rev transactivator in cells transfected with pNLgCAT was in good

Table 1
Enhancement of cytoplasmic mRNA expression by Rev transactivator

mRNA length	Rev		Ratio (+/−)
	+	−	
Full	1732	36	48
Medium	2454	336	7.4
Short	5128	5692	0.9

A Rev-expression vector prevl [21] (+), or its backbone vector pRVSV [21] (−), was contransfected with a Rev-defective proviral clone pNL-Ba [8], and 2 days later RNA was prepared as described in section 2. Northern blot hybridization was performed as shown in Fig. 1B and radioactivity of the bands corresponding to three major species of HIV-1 mRNAs was measured by bio-imaging analyzer BAS2000. Data are presented in PSL units of this system, which are proportional to cpm units. Results are presented as an average of three independent experiments.

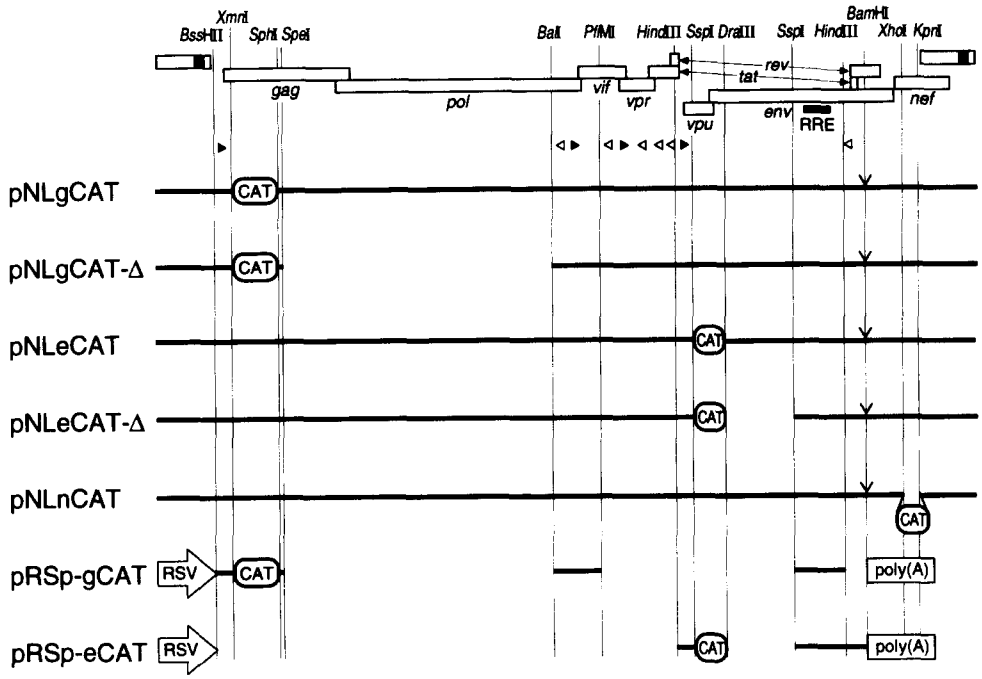

Fig. 2. Structures of reporters to monitor Rev activity. Each reporter contains CAT gene as a marker gene. The genome structure of an infectious DNA clone of HIV-1, named pNL432 [25] and the recognition sites of restriction endonucleases used for DNA manipulations are shown at the top. Closed and open arrowheads indicate major donor and acceptor signals for mRNA splicing, respectively [22]. Sequences contained in the reporters (bold line) and the position of CAT gene inserted into the reporters are also indicated. *rev* gene of each pNL reporter is disrupted by a frame-shift mutation at the *Bam*HI site [8]. RSV promoter and SV40 poly(A) signal are represented by open arrows and open boxes, respectively.

agreement with that of virion production of Rev-deficient virus by Rev previously reported by us [8].

By using these reporter clones, we determined whether the differential regulation of HIV-1 gene expression by Rev is commonly observed in various cell lines. Several cell lines derived from primates, rodents and feline were transfected with CAT-containing reporter clones in the presence or absence of Rev, and expression of CAT activity was examined. In all cell lines tested, generally similar results with those in SW480 cells were obtained (Table 2). There was no clear cell-specificity with respect to Rev-responsiveness. However, in cell lines U937 and SC-1, expression of CAT directed by pNLgCAT and pNLeCAT was very poor even in the presence of Rev. Worthy of note is that CAT production by pNLnCAT was also very low in SC-1 cells. These results suggested that there was some cell-specific dependency of HIV-1 mRNA expression on Rev.

3.3. Determinant for differential regulation by Rev transactivator

To determine the location (*gag-pol* and/or *vpu-env*) of element(s) responsible for the differential regulation by Rev transactivator presented above, deletion analysis of CAT-containing reporters was performed. We constructed pNLgCATΔ and pNLeCATΔ, which have large deletions in *gag-pol* and *env* coding sequences, respectively (Fig. 2), and cotransfection experiments in SW480 cells were carried out using these reporters. As shown in Fig. 3, these deletions had no influence on the Rev-responsiveness of parental reporter clones. These results

Table 2
Transactivation of reporter CAT constructs by Rev protein in various cell lines

CEll	pNLgCAT			pNLeCAT			pNLnCAT		
	+	−	Ratio	+	−	Ratio	+	−	Ratio
SW480	58.7	1.4	42	76.4	22.1	3.5	86.2	92.6	0.9
COS7	43.4	0.5	87	24.9	3.0	8.3	35.4	64.5	0.5
A3.01	34.3	0.5	69	71.7	14.5	4.9	74.2	88.1	0.8
U937	0.7	*	**	4.4	0.6	7.3	23.4	54.5	0.4
SC-1	0.5	*	**	1.1	0.5	2.2	8.0	5.8	1.4
CRFK	43.7	0.7	62	71.4	12.0	5.9	81.7	86.5	0.9
BHK	19.8	0.4	50	21.5	2.2	9.8	59.6	47.1	1.3

CAT-containing viral reporters were transfected into various cell lines with a Rev-expression vector prevl [21] or its backbone plasmid pRVSV [21]. Two days after transfection, CAT activity in the transfected cells was monitored. Percent conversion of chloramphenicol to its acetylated forms in the presence (+) or absence (−) of Rev protein, and transactivation ratio by Rev (+/−) are presented. *Background level of conversion (<0.3%); **ratio can not be determined because (−)-value is too low.

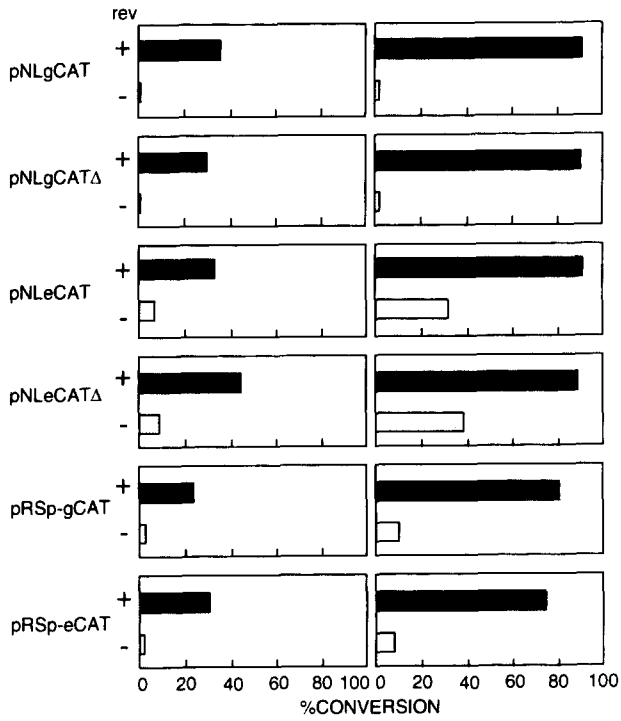

Fig. 3. Rev-responsiveness of various reporter constructs. Each of reporter constructs (1 μg) was transfected into SW480 cells with a Rev-expression plasmid prev1 (10 μg) (+) or its backbone plasmid pRVSV (10 μg) (−). Two days after transfection, cell lysates were prepared and CAT activity was determined. Percent conversion of chloramphenicol to its acetylated forms is shown. Left and right panels represent results obtained by 0.5 h and 3 h reaction time for CAT assay, respectively.

clearly indicated that almost all *cis*-elements located in *gag-pol* and *env* coding sequences did not serve as the determinants for differential regulation by Rev regulatory protein. Furthermore, RT production in culture supernatants of SW480 cells transfected with pNLeCAT or pNLeCATΔ was drastically enhanced by Rev transactivator in a similar manner (data not shown).

For more detailed analysis of the determinants for Rev-responsiveness, we constructed simple reporters designated pRSp-gCAT and pRSp-eCAT (Fig. 2). Expression of marker CAT gene of these clones was driven by RSV promoter, and most of the *gag-pol* and *env* coding sequences of pNLgCATΔ and pNLeCATΔ were removed from the two constructs (Fig. 2). We have previously demonstrated that the regions containing splice signals of pRSp-eCAT had an important role for Rev-functionality [21]. pRSp-gCAT and pRSp-eCAT were constructed so as to retain the splice signals. These CAT-reporters were transfected into SW480 cells with or without a Rev expression plasmid, and CAT production was monitored. As shown in Fig. 3, Rev-responsiveness of the two reporters still remained, but the extent of activation of the clones by Rev was similar. These results showed that structural difference between pNL and pRSp constructs was important for the differential regulation by Rev regulatory protein, but not for Rev-functionality.

4. Discussion

We reported here that expression of HIV-1 genes was differentially regulated by viral transactivator, Rev. This is the first report on comparative analysis of Rev-responsiveness of three species of HIV-1 mRNAs. As clearly shown in this report, expression of *gag-pol* gene and genomic RNA was highly dependent on *rev* gene function, but those genes encoded for by medium-length mRNAs showed relatively low dependency (Fig. 1, Fig. 3, Table 1 and Table 2). This phenomenon was generally observed in a wide variety of cell lines (Table 2). These results suggested that virion production of HIV-1, which is mainly determined by *gag-pol* gene expression, is strictly controlled by Rev and that *env* and other genes encoded for by medium-length mRNAs can be partially expressed without Rev. Whether viral proteins are actually translated from these medium-length mRNAs in the absence of Rev in HIV-1 replication cycle needs to be determined.

The determinant(s) for the difference between dependencies of expression of full- and medium-length mRNAs on Rev function was studied by using various CAT constructs (Fig. 2). One possibility was that some *cis*-elements in *gag-pol* and *vpu-env* coding sequences have a crucial inhibitory effect on accumulation of mRNA in cytoplasm, and that the difference of the effect resulted in the distinct Rev-responsiveness of mRNAs. This is unlikely from the results that pNLgCATΔ and pNLeCATΔ had essentially the same Rev-responsiveness with the parental reporters, pNLgCAT and pNLeCAT (Fig. 3). Reporters pNLgCATΔ and pNLeCATΔ had large deletions in *gag-pol* and *vpu-env* coding sequences, respectively, and almost all

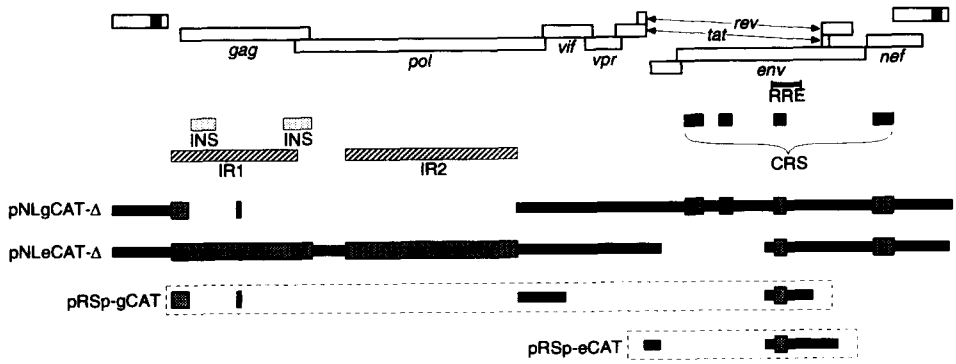

Fig. 4. *Cis*-acting inhibitory sequences and reporter clones used in this study. The location of *cis*-acting repression sequences designated as CRS [14,15], INS [16,17] and IR [18] are indicated. The sequences contained by some reporters used in this report are indicated by thick lines. The overlapping regions of reporters to *cis*-acting repression sequences are represented by bold dotted boxes.

presumed *cis*-elements [14–18] were removed (Fig. 4). Another possibility was that splice signals for removing out *gag-pol* and *vpu-env* sequences have different effects on accumulation of mRNAs in cytoplasm and confer distinct Rev-dependency on mRNAs. To test this, we constructed two simple reporters, designated pRSp-gCAT and pRSp-eCAT (Fig. 2). These reporters contained the splice signals for excluding exons of *gag-pol* and *vpu-env* genes, respectively. These two reporters responded in a similar way to Rev protein (Fig. 3). From these results, the splice signals present in pRSp-gCAT and pRSp-eCAT appeared not to contribute to differential response of mRNAs to Rev transactivator. Taken together, the differential regulation by Rev seemed to be determined not by *cis*-elements in *gag-pol* and *vpu-env* coding sequences, but by a fundamental viral structure remaining in both pNLgCATΔ and pNLeCATΔ.

Deletion analysis described above also gives an insight into Rev action. It has been postulated that CRS [14,15], IR [16,17] and INS [18] prevent transport of full- and medium-length mRNAs into cytoplasm and that these elements confer Rev-dependency on these mRNAs. Although almost all of these elements were removed from our reporter clones pRSp-gCAT and pRSp-eCAT (Fig. 4), they still retained Rev-responsiveness (Fig. 3). Both pRSp reporters contained one of CRS's overlapping RRE [13,14], but we previously showed that a fragment containing RRE only was not sufficient to confer Rev-responsiveness on mRNAs [21]. A portion of IR1, which carries the major splice donor, was contained in pRSp-gCAT. The IR1 was reported to be an inhibitory region for gene expression but its significance for Rev functionality was unclear [18]. In addition, a small part of the IR1 had no apparent inhibitory effects on gene expression [18]. Thus, the possibility that this IR1 fragment contributed to the Rev-responsiveness of pRSp-gCAT seemed to be unlikely. In contrast, our previous study on pRSp-eCAT [21] showed the importance of splice signals for Rev-functionality. On the basis of these results, it is likely that Rev-requirement for expression of mRNAs is conferred by splice signals rather than by *cis*-acting inhibitory sequences.

It is worthy to note that full-length mRNA has more splice signals than medium-length mRNAs. Therefore, the former may show more dependency than the latter for its expression. Full-length mRNA of simple retroviruses such as murine leukemia virus has a structure similar to that of the medium-length mRNAs of HIV-1. These viruses can express whole sets of mRNAs without Rev-like function.

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