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Modulation of HTLV-I Gene Expression by HIV-1 Rev through an Alternative RxRE-Independent Pathway Mediated by the RU5 Portion of the 5′-LTR

Satoshi Kubota, Rika A. Furuta,*[,1] Masakazu Hatanaka,*[,2] and Roger J. Pomerantz[3]

The Dorrance H. Hamilton Laboratories, Center for Human Virology, Division of Infectious Diseases, Department of Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107; and
**Institute for Virus Research, Kyoto University, Sakyo-ku Kyoto 606-01, Japan*

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The 5′-RU5 portion of human T-lymphocyte virus type I (HTLV-I) long terminal repeat (LTR) had been reported to contain *cis*-acting elements for the controlled viral gene expression by the *rex* gene product. In this study, the human immunodeficiency virus type I (HIV-1) Rev protein was found to enhance gene expression, acting through the 5′-RU5 portion of HTLV-I, while the Rex-responsive element (RxRE)-mediated activation by Rev was reconfirmed to be negative. This positive action of HIV-1 Rev on HTLV-I gene expression seemed to be distinct from the widely accepted Rex or Rev function to facilitate the nuclear export of RxRE-containing unspliced viral mRNAs, since a *trans*-dominant, nuclear export-deficient mutant (RevM10) still retained the RU5-mediated effector function. Analyses of the functional aspects of Tat/Rev fusion proteins on the HTLV-I RU5 suggested a specific interaction of Rev and RU5, but lacked evidence for the binding of Rev to the RU5 at the RNA level. These results suggest an answer to the controversy regarding a Rex-like function occasionally observed with HIV-1 Rev and its related proteins. It may also be suggested that particular care should be taken when such a *trans*-dominant Rev mutant is considered to be used as a genetic therapy against HIV-1 infection, in individuals infected with both HIV-1 and HTLV-1. © 1998 Academic Press

The Rev protein of human immunodeficiency virus type 1 (HIV-1) and the Rex protein of human T-cell leukemia virus type I (HTLV-I) are heterologous viral regulatory proteins of similar functions. They are localized predominantly in the nucleoli in cells expressing these proteins (1-4), shuttling between the nucleus and cytoplasm under the cooperation of their own nuclear/nucleolar targeting signals (NOS) (1,3) and nuclear export signals (NES) (5-12). Rev, or Rex, binds to a structured target on viral unspliced and partially-spliced mRNA, which is entitled Rev-, or Rex-responsive element (RRE or RxRE, respectively) (13-18). Therefore, the nucleo-cytoplasmic shuttling property is believed to play a key role in the observed Rev, or Rex, function to enable the cytoplasmic accumulation and efficient translation of the mRNAs for viral structural and enzymatic proteins, together with the RRE, or RxRE-mediated interaction.

It is known that the Rex protein binds to RRE as well as RxRE *in vitro*, and acts as an exogenous 'Rev' protein for HIV-1 (3,16,19-22). In contrast, Rev does not bind to RxRE *in vitro*, (17,18) and no detectable RxRE-mediated transactivation function has been reported (19-21). However, several reports have described a transactivator function of Rev in some Rex function assay systems (3,23), which possess certain extra portions of HTLV-I sequence, including the 5′-RU5 sequence, as well as the RxRE. Interestingly, the 5′-RU5 segment has been shown to contain two *cis*-acting elements for viral gene expression (24,25), which also seem to be required to confer full Rex-responsiveness cooperating with RxRE in the 3′-U3R (25). In this report, the responsive region for the modulation effect of Rev on HTLV-I gene expression is mapped, not in the RxRE, but in the RU5 region of the 5′-LTR, which suggests an accessory function of Rev, apart from the major RRE-mediated transactivating function.

MATERIALS AND METHODS

Cell cultures. Cos-1 cells and HeLa cells were maintained in Dulbecco's modified minimum essential medium (DMEM) supplemented

[1] Present address: Food and Drug Administration, Center for Biologics Evaluation and Research, Office of Vaccines Research and Review, Division of Allergenic Products and Parasitology, Rockville, MD 20852-1448.

[2] Present address: Shionogi Institute for Medical Science, Settsushi, Osaka, 566, Japan.

[3] Corresponding author. Fax: (215) 923-1956. E-mail: rpomvic1@jeflin.tju.edu.

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by 10% fetal bovine serum (FBS) in humidified air containing 5% CO_2 at 37°C.

HIV-1 Rev expression plasmids and control plasmids. The plasmids pH2rev (3) and pH2RexdL (12) have been described previously. Also, pcREV, pcTAT, pcTAT/REV, and pcTAT/M10, which were described in previous studies (4,26), have been kindly provided by Dr. B.R. Cullen. Construction of pH2revM10 was carried out by subcloning the pcREVM10 Sac I-Hind III fragment, which encodes a Rev mutant M10, into the unique Hind III site of pKCRH2 (3), the parental mammalian expression vector for all the constructs shown in Figure 1A. The other Rev mutant expression plasmid, pH2trev, has been constructed by introducing a frame-shift mutation at the internal BamHI site by enzymatic digestion and filling ends in pH2rev, followed by self ligation. Structures of these mutants are illustrated in Figure 1A.

Reporter constructs. Rex function-reporter plasmids, pCATx and pSPLB15, have been described previously (3,23). A derivative of pCATx, entitled pCATxdL, was constructed by removing the SpeI-BamHI 1.05kb fragment containing the entire 3′LTR by partial enzymatic digestion and religation. The other reporter plasmids, pRSpCATRXE, and pSLIIB/CAT, were kindly provided by Drs. A. Adachi and B.R. Cullen, respectively (21,26).

DNA transfection and chloramphenicol acetyl transferase (CAT) assay. Five micrograms of each effector plasmid and 2.5μg (pCATx, or pRSpCATRXE), or 1μg (pCATxdL) of each reporter plasmid were co-transfected into COS7 cells by a DEAE-dextran method, as previously described (27). For HeLa cells, a liposome-mediated DNA transfer technique was applied, using a commercial kit (LipofecTAMINE: GIBCO/BRL) under the manufacturer's optimized protocol. In those experiments, 800 ng of pCATx, or pSLIIB/CAT, and 200 ng of pcTAT/REV, pcTAT/M10, pcTAT, or pcREV were co-transfected into HeLa cells. After 48h, cells were harvested and their lysate was prepared in 0.25M Tris-Cl pH 8.0. Measurement of CAT activity was carried out as described previously (3,28).

Percent conversion values were calculated by quantitating each spot of radioactive chloramphenicol on the thin layer chromatography (TLC) plate. Quantification was carried out by a phosphorimager (Molecular Dynamics) with the TLC plates, or by a densitometer (Molecular Dynamics) utiilzing the autoradiograms.

RESULTS

Mapping of the cis-Acting Element in HTLV-I, Which Is Responsive to HIV-1 Rev

The transactivation of HTLV-I gene expression by HIV-1 Rev was initially determined by a Rex function reporter construct of [LTR-CAT-U3R] type (pSPLB15 : Figure 1B). Since it has been reported that Rev does not bind directly to RxRE, we hypothesized a different mechanism and pathway of the observed effect of Rev from the classical Rex or Rev function. In order to specify which region in the HTLV-I LTRs in pSPLB15 is responsible for the activation by Rev, three additional CAT reporter constructs were obtained, and the effect of HIV-1 Rev on CAT gene expression was evaluated further. The results are illustrated in Figure 2 and Table 1. Of note, a Rex functional reporter, entitled pRSpCATRXE, completely lacked responsiveness to Rev expression (Figures 1B, 2C and Table 1). In contrast, another Rex reporter construct, pCATx, which contains 5′-RU5 and 3-LTR as the common elements with pSPLB15, demonstrated a positive response to the Rev expression, similar to pSPLB15 (Figure 1B, 2A and

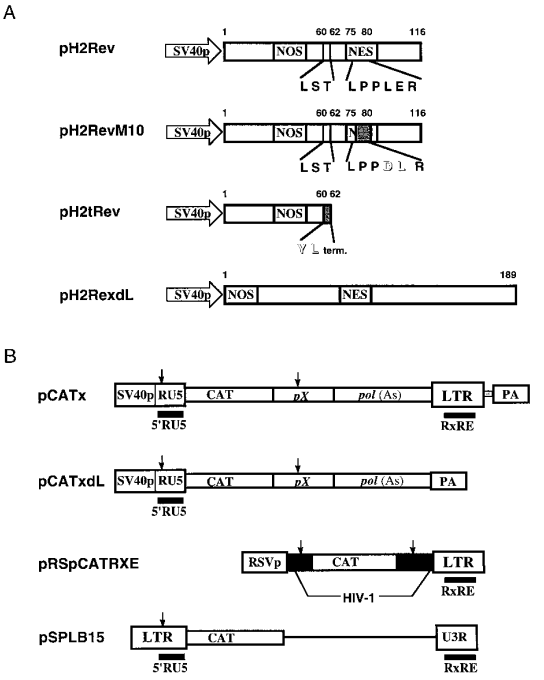

FIG. 1. (A) Structure of expression plasmids. Large boxes represent the primary structures of the proteins to be expressed. Numbers over these boxes denote residue numbers counted from the initiation methionines. Approximate location of the nuclear/nucleolar targeting signals (NOS) and nuclear export signals (NES) are shown as small boxes with abbreviations. Under the boxes which represent Rev and its mutants, amino acid residues around the mutated areas are displayed as single letter codes, in which mutated residues are represented in shaded letters and "term" for the introduced termination codon. Positions of mutated areas are shown as shaded boxes. SV40p is an abbreviation for an SV40 promoter. (B) Structure of reporter plasmids. Three plasmids in this panel had been described previously in detail. Abbreviations; SV40p: SV40 promoters, RSVp: Rous sarcoma virus LTR, RU5: HTLV-I RU5 segments, CAT: bacterial CAT gene, *pX:* fragments from HTLV-I *pX* region, *pol* (As): fragments from HTLV-I *pol* gene in antisense orientation, PA: SV40 polyadenylation signals. LTR: HTLV-I LTR, U3R: HTLV-I U3R segment. Arrows denote viral splice donor and acceptor sites. Black boxes are HIV-1-derived fragments containing intact splice donor and acceptor sites. Locations of 5′-RU5 and RxRE are indicated by thick black lines under each illustration.

Table 1). Interestingly, pCATxdL, which is a derivative of pCATx with the 3′-LTR (containing RxRE) deleted, still retained the Rev-responsiveness, although it lost RxRE and the proper response to Rex expression, compared to pCATx (Figure 1B, 2B and Table 1). These results, and previous data on pSPLB15, are summarized in Table 2. As clearly shown, the only common region which is shared by all the Rev-responsive constructs, and which does not exist in the Rev-unresponsive construct, is the RU5 of the HTLV-I 5′-LTR.

Nuclear Export of Rev Is not Required for Its Transactivating Function through HTLV-I 5′-RU5

The HIV-1 Rev mutant, M10, has been described as a typical *trans*-dominant mutant; hence it inhibits Rev

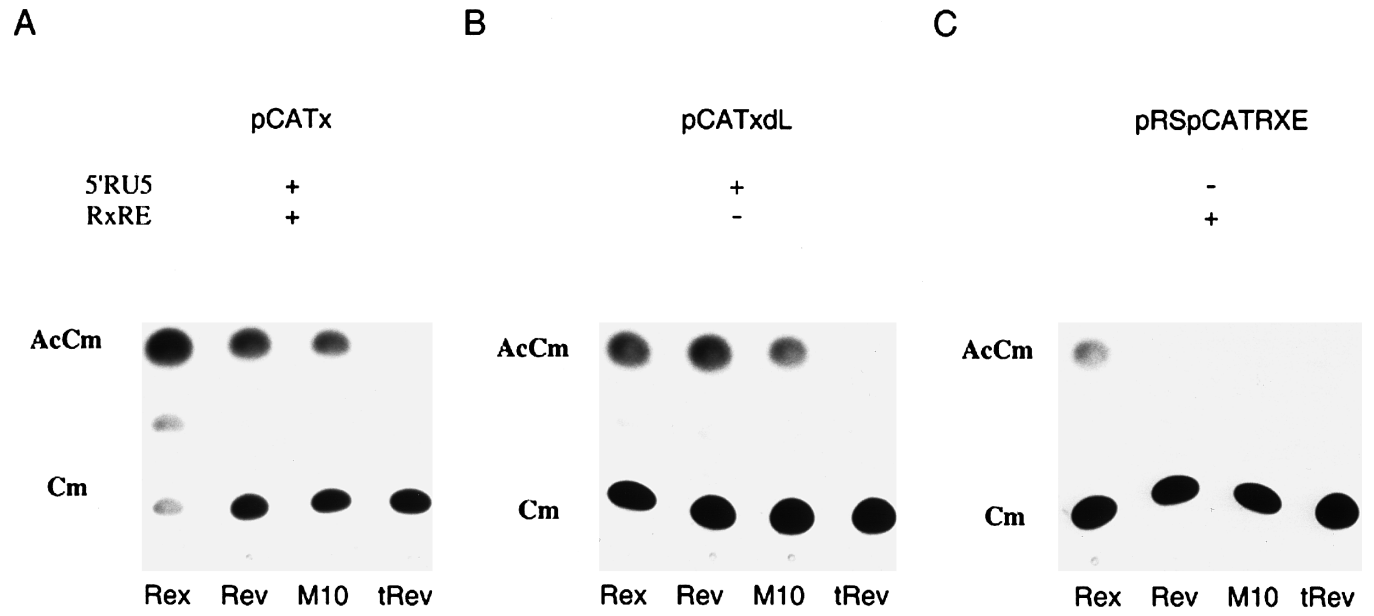

FIG. 2. Effect of Rev and related proteins on the *cis*-acting elements of HTLV-I. Each expression plasmid displayed in Figure 1A was co-transfected into Cos 7 cells with (A) pCATx, (B) pCATxdL, or (C) pRSpCATRXE, then expressed CAT activity was evaluated, using 5μl (A and B), or 20μl (C) out of 60μl of total lysate. Reactions were performed at 37°C for 20 min (A), 2 h (B), or 4 h (C). The involvement of *cis*-acting elements in each reporter construct is shown between the name of the reporter and photographs. Positions of 3-acetylated and non-acetylated forms of chloramphenicol are indicated at the left of each panel. Names of expressed Rev and related proteins are shown at the bottom. A mutant, tRev, serves as a negative control. These data are representative of at least three independent experiments.

function and it retains no Rev function. Since the mutation inscribed in M10 is located in the nuclear export signal-encoding region, it is defective in nucleocytoplasmic shuttling (11), which is thought to be one of the major determinants for its dominant-negative phenotype (4). Interestingly, however, M10 was found to still retain the transactivating property through the 5′-RU5 of HTLV-I. As shown in Figure 2 and Table 1, M10 behaved almost in the same manner as wild-type Rev, as evaluated in our three different reporter systems. These results indicate that nuclear export is not required for the HTLV-I 5′-RU5-mediated transacting function of HIV-1 Rev, and also strongly suggest a different mechanism for the observed transactivation by Rev, from the well-characterized Rev or Rex function mediated by RRE or RxRE.

TABLE 1

Quantitative Analysis of CAT Assays

	% Conversion			
	Rex	Rev	RevM10	tRev
pCATx	87.4	49.7	38.4	5.1
pCATxdL	26.9	28.5	18.0	3.8
pRSpCATRXE	15.0	3.4	2.6	1.7
	% Conversion			
	Tat/Rev	Tat/M10	Tat	Rev
pSLIIB/CAT	30.1	21.2	0.3	0.5
pCATxdL	14.3	15.5	1.1	12.8

Note. The results displayed in Figs. 2 and 3 were analyzed and summarized.

Functional Comparison with HIV-1 Tat Function, Which Is Mediated by Heterologous RNA–Protein Interaction

The RU5 segment at the 5′-end of HTLV-I mRNA is also predicted to form a stable secondary structure that is different from the RxRE in the 3′-U3R (Figures 3A and 3B). The involvement of the structured element

TABLE 2

Summary of the Rev-Responsiveness of the Reporter Constructs, in Comparison with the Involvement of HTLV-I Segments in Each Construct

	5′-RU5	*px-pol*	RxRE	Rev-responsiveness
pCATx	+	+	+	+
pCATxdL	+	+	−	+
pRSpCATRXE	−	−	+	−
pSPLB15	+	−	+	+

Note. The Rev-responsiveness of pSPLB15 had been described previously (3). The concordance between the Rev responsiveness and the involvement of 5′-RU5 is emphasized by boldface type.

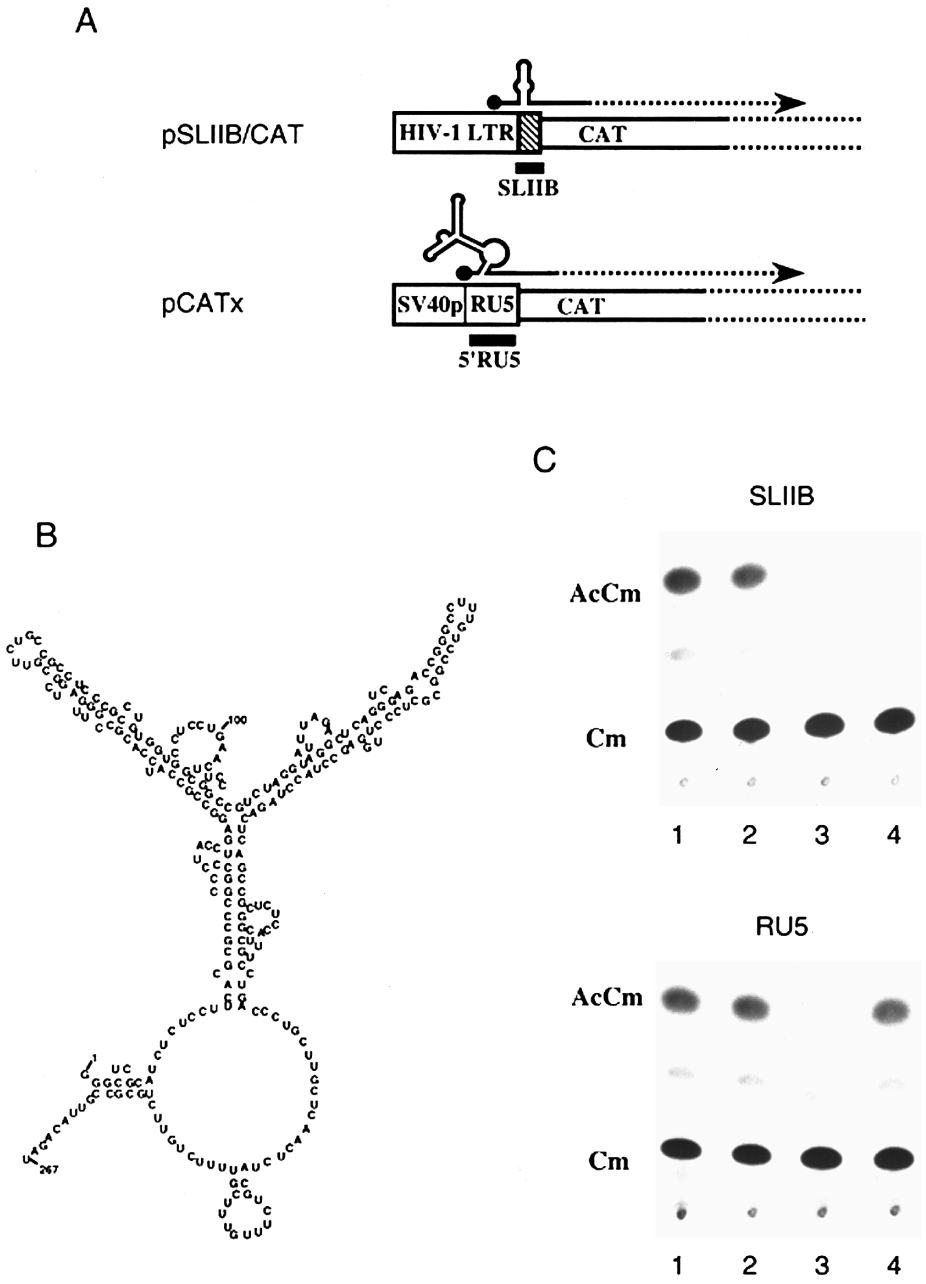

FIG. 3. (A) Schematic comparison of the structures of pSLIIB/CAT and pCATx and their transcripts. The transcripts are illustrated at the top of each DNA construction with predicted secondary structures. Arrows denote the direction of transcription. Solid circles denote the cap structures of mRNAs. SLIIB (with a crossed small box) represents a minimal high-affinity binding site of Rev in RRE. Other abbreviations represent the same items as described in Figure 1B. (B) A predicted secondary structure of HTLV-I 5′-RU5 mRNA segment. Displayed numbers for some nucleotides are counted from the cap site. The structure was predicted by a program in the DNASIS package (HITACHI SK). (C) Comparative analysis of the responsiveness to Tat, Rev, and their fusion proteins between pSLIIB/CAT and pCATx. Names of reporter constructs and positions of acetylated and non-acetylated forms of chloramphenicol are shown as in Figure 2. Lane 1: pcTAT/REV. Lane 2: pcTAT/M10. Lane 3: pcTAT. Lane 4: pcREV. Reactions were performed at 37°C for 2 h with each lysate containing 2.5 μg of total protein. These data are the representative of at least two independent series of experiments.

immediately downstream of the transcriptional start site is reminiscent of the 5′-structure of the HIV-1 mRNA which involves the TAR element as the Tat binding site. Therefore, we suspected a Tat-like transactivation mechanism in the observed transactivation via RU5. As a control reporter plasmid, another CAT-based construct, pSLIIB/CAT, was utilized. In this plasmid, the CAT gene is driven by HIV-1-LTR in which the TAR element has been replaced by the high-affinity binding site for Rev. Transactivation of this

chimeric promoter is carried out by a Tat/Rev fusion protein under the cooperation of RNA binding by the Rev segment and the transcriptional activation by the Tat segment. Tat/Rev and Tat/M10 displayed expected transactivation on this promoter, whereas neither Tat nor Rev alone could transactivate this construct. Using the same fusion constructs, the response of pCATxdL was tested in a similar manner. As shown in Figure 3C and Table 1, both fusion proteins yielded transactivation, while Tat showed no significant levels of CAT gene expression, serving as a negative control. However, on pCATxdL, Rev itself gave rise to significant transactivation, again confirming the results in Figure 2B, and suggesting a different mechanism of transactivation from the RNA-linked transcriptional activation by HIV-1 Tat.

DISCUSSION

In the present study, the 5′-RU5 portion of HTLV-I has been found to contain a *cis*-acting element, through which HIV-1 Rev acts as a positive *trans*-regulator. Apart from our present results, the RU5 portion has been previously described to possess unique *cis*-acting elements for viral gene expression. The earliest study of this matter uncovered the involvement of a *cis*-activator for gene expression (24). This enhancing effect by 5′-RU5 was, interestingly, orientation-dependent, which is unlike prototypic DNA enhancers for transcription. Another study described a repressive element in the 5′-U5 region, which was shown to be a *cis*-acting element other than RxRE, for the full-Rex responsiveness of the regulated expression of unspliced viral mRNA (25). Furthermore, a later study identified a short DNA sequence at the boundary of R and U5 which regulated HTLV-I basal gene expression at the transcriptional level, through the binding of cellular factor(s) to the corresponding DNA segment (29). Therefore, the 5′-RU5 of HTLV-I seems to be a composite of several *cis*-acting elements, hence the mechanism of the observed Rev responsiveness herein is quite hard to fully dissect at present. However, it is at least evident that the observed Rev action on the RU5 is distinct from the classical Rev function through the RRE; for the shuttling-deficient mutant M10, that is totally nonfunctional as Rev, also gave a similar level of transactivation through the RU5. These data also suggest that the RU5-mediated transactivation should be a nuclear event.

Taken together, HIV-1 Rev seems to interact, directly or indirectly, with the DNA or RNA of the RU5 segment to enhance gene expression, either by increasing the level of transcription, or inhibiting splicing. Since the RU5 portion of unspliced HTLV-I mRNA is predicted to form a stable secondary structure, direct binding of Rev to this structure was suspected to be a primary interaction of Rev and the RU5. In this context, the effect of Tat/Rev fusion proteins were examined to estimate this possible interaction. Since the activation function involved in Tat has been shown to be as general and effective as the VP16 activation domain (30), if Rev binds directly to the RU5 RNA, potent transactivation can be expected by Tat/Rev fusion constructs. However, the Tat/Rev fusion protein yielded as much effect as Rev alone on the RU5. As such, binding of Rev to the RU5 RNA seems to be unlikely at present. In addition, no positive data for *in vitro* binding of Rev to RU5 has been obtained in our studies.

In terms of the action of the HTLV-I Rex protein, an interesting finding is observed on pCATxdL. Although the RxRE in the 3′-LTR had been entirely removed, this reporter still displayed some Rex responsiveness. However in a previous report, a construct with a CAT gene driven by the HTLV-I-LTR was shown to be unresponsive to Rex expression (31), which was also confirmed in our studies with a similar LTR-CAT construct (data not illustrated). The major difference between these LTR-CAT constructs and pCATxdL is the presence of an intact HTLV-I splice acceptor site downstream of the CAT gene of pCATxdL. Interestingly, a similar reporter construct of HTLV-II with 5′-RU5 (involving the major splice donor site) in combination with viral splice acceptor site is responsive to the Rex protein of HTLV-II (Rex II), which resulted in the definition of two RxREs in HTLV-II unspliced mRNA - one of which in the 5′-RU5 and the other in the 3′-LTR (32,33). Thus, the existence of a minor "RxRE" in the 5′-RU5 of HTLV-I, which requires both splice donor and acceptor of intact origin for detectable activity, may be hypothesized. Indeed, it is at least clear that also in HTLV-I, the 5′-RU5 is required for an efficient response to Rex, in cooperation with the RxRE at the 3′-LTR (25). Analyses at the mRNA level are ongoing.

The effect of HIV-1 Rev on HTLV-I gene expression has been controversial. Some reports showed a "Rex-like" enhancement of gene expression by Rev, using CAT-based reporters with 5′-RU5 and RxRE of HTLV-I (3,23), while no such activity was observed in other studies in which Rex function reporters containing RxRE only (without 5′-RU5) were used (19-21). The result obtained in the present study suggests an answer to account for those previous findings, which are apparently contradictory. The effect of HIV-1 Rev on HTLV-I gene expression is not an RxRE-mediated "Rex-like" effect, but an alternative effect through an element in the RU5, the precise mechanism of which remains yet to be clarified.

Along with the prevalence of HIV-1, the number of co-infected cases with HIV-1 and HTLV-I have been increasing. To combat against HIV infection, several gene therapeutic approaches have been developed, which include ones that are targeted to Rev function. Among them, the most advanced Rev-based anti-HIV-1 gene therapeutic strategy utilizes the *trans*-dominant

mutant, M10, as an anti-Rev agent (4,34). This strategy seems to be a fairly promising way to combat HIV-1. However, in those cases with HTLV-I infection, particular caution should be taken in utilizing the M10 mutant, since it may cause the stimulation of HTLV-I provirus, which results in non-functional proliferation of CD4+ lymphocytes and possibly, early onset of malignancy (35,36). Utility of potent inducible M10 expression vectors (37), rather than constitutive ones, are especially useful for the application of such an anti-HIV-1 strategy in HTLV-I carriers at the risk of AIDS.

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