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Two cis-acting elements responsible for posttranscriptional trans-regulation of gene expression of human T-cell leukemia virus type I

(*pX* region/*rex* gene function/mRNA splicing/$p27^{rex}$ target sequence)

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ABSTRACT **The *pX* sequence of human T-cell leukemia virus type I codes for two nuclear proteins, $p40^{tax}$ and $p27^{rex}$, and a cytoplasmic protein, $p21^{x\text{-}III}$. $p40^{tax}$ activates transcription from the long terminal repeat (LTR), whereas $p27^{rex}$ modulates posttranscriptional processing to accumulate *gag* and *env* mRNAs that retain intron sequences. In this paper, we identify two cis-acting sequence elements needed for regulation by $p27^{rex}$: a 5′ splice signal and a specific sequence in the 3′ LTR. These two sequence elements are sufficient for regulation by $p27^{rex}$; expression of a cellular gene (metallothionein I) became sensitive to *rex* regulation when the LTR was inserted at the 3′ end of this gene. The requirement for these two elements suggests an unusual regulatory mechanism of RNA processing in the nucleus.**

Human T-cell leukemia virus type I (HTLV-I) (1, 2) is the etiologic agent of adult T-cell leukemia (2–5), and its genome contains an extra region, called *pX* or *X*, between the *env* gene and the 3′ long terminal repeat (LTR) (6–9). This region contains multiple genes and is expressed as a doubly spliced mRNA (ref. 10 and Fig. 1) that encodes three proteins by using two overlapping open reading frames, ORFs IV and III (11): $p40^{tax}$ derives from ORF IV (12–15) and $p27^{rex}$ and $p21^{x\text{-}III}$ derive from ORF III (16). Both $p40^{tax}$ and $p27^{rex}$ are trans-acting regulators localized in the nucleus (16).

$p40^{tax}$, a protein of 40 kDa, is a trans-acting factor that activates viral gene transcription from the HTLV-I LTR (17–21). $p40^{tax}$ was also found to activate expression of the cellular genes for interleukin 2 and its receptor in certain T-cell lines (22) by enhancing its promoter activity (23–25). Thus $p40^{tax}$ may account for uncontrolled proliferation of HTLV-I-infected T cells (26, 27).

$p27^{rex}$ is required for *gag* gene expression and is thought to operate at the posttranscriptional level (28–30). The *rex* function was first demonstrated by use of a largely deleted provirus. In the absence of $p27^{rex}$, $p40^{tax}$ alone activated transcription of the provirus, but all transcripts were spliced into smaller mRNA, and *gag*-encoded protein was not expressed. However, the coexpression of $p27^{rex}$ and $p40^{tax}$ induced accumulation of unspliced *gag* mRNA (28, 29). Further studies using a full-sized proviral DNA carrying a mutation in the $p27^{rex}$ coding sequence showed that accumulation of both *gag* and *env* mRNAs was dependent on the presence of $p27^{rex}$ (30).

Here we report that two cis-acting elements are required for regulation by $p27^{rex}$: a 5′ splicing signal and a specific sequence of the 3′ LTR. These two elements must be transcribed into target RNA for *rex* regulation. The requirement for elements in both the 5′ and the 3′ region suggests an unusual posttranscriptional mechanism of $p27^{rex}$.

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Recently proposed (38) terms for the trans-acting regulators of HTLV are used: *tax* for ORF IV, which encodes $p40^{x}$, and *rex* for ORF III, which encodes $p27^{x\text{-}III}$.

MATERIALS AND METHODS

Plasmids, Transfection, and Analysis of mRNA. pMTCXds-ATG1&4 and pMTCXsn-IV^{ter} are expression plasmids for $p40^{tax}$ and $p27^{rex}$, respectively. Expression is directed by the metallothionein (MT) promoter (28, 29). Transfection of the plasmids was carried out as described (19). In brief, 7×10^5 cells of the human amnion cell line FL were transfected with 15 μg of a mixture of plasmid and *Escherichia coli* DNAs by the calcium phosphate method. After 40 hr, the cells were harvested, and poly(A)$^+$ RNA was subjected to blot hybridization analysis (28).

Assay for Chloramphenicol Acetyltransferase (CAT) Activity. Forty hours after transfection, total cell extracts were prepared by three cycles of freezing and thawing followed by brief centrifugation. Samples containing 30 μg of protein were used for assay of CAT activity at 37°C (19). Under these conditions, the activity was proportional to protein concentration and to incubation time.

RESULTS

Two Cis-Acting Elements Required for *rex* Regulation. We have reported (29) that *rex* function was required for expression of *gag* protein by a defective HTLV-I construction, pGAGdm, that contained the intact *gag* gene between the two HTLV-I LTRs (Fig. 2*A*). Without *rex*, only spliced mRNA was detected; thus, no *gag* protein was expressed. Complementation with an expression plasmid containing the *rex* gene induced accumulation of the unspliced form of mRNA and concomitant expression of *gag* protein. However, splicing itself seemed not to be necessary for *rex* function, because the target RNA was also accumulated even without the 3′ splice signal when *rex* expression plasmid was present; otherwise the RNA was not detectable. Therefore, the *rex* gene product can induce accumulation of the target RNA regardless of whether that RNA can be normally spliced (29).

For determination of cis-acting sequences required for *rex*-dependent accumulation of RNA, the *gag* sequence and the 3′ splice signal in pGAGdm were replaced by the bacterial CAT gene to construct pL-CAT-L (Fig. 2*A*), and extracts of transfected cells were assayed for CAT RNA and enzyme activity. A *tax* expression plasmid was always cotransfected

Abbreviations: HTLV-I, human T-cell leukemia virus type I; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; ORF, open reading frame; MT, metallothionein; TK, thymidine kinase; m.p., map position(s).

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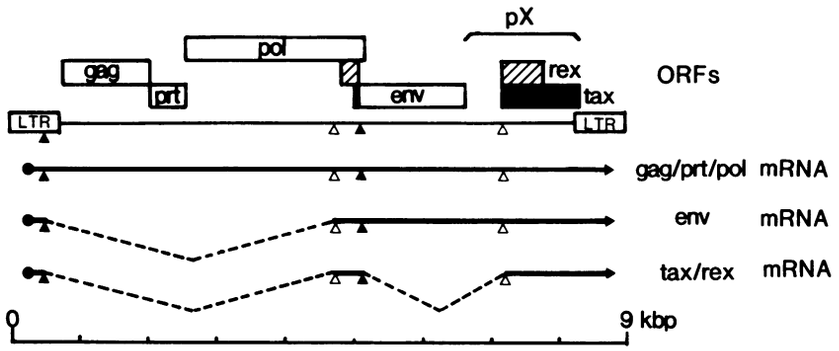

FIG. 1. HTLV-I gene expression. Open boxes represent open reading frames (ORFs) in the HTLV-I genome. Horizontal lines with arrowheads represent viral mRNA for *gag/pol, env*, and *tax/rex*. ▲ and △, 5′ and 3′ splice signals, respectively. kbp, Kilobase pairs.

to maintain efficient transcription from pL-CAT-L. Cotransfection of a *rex* expression plasmid resulted in a dose-dependent increase in CAT activity (Fig. 2*B*) and an almost parallel increase in CAT mRNA (Table 1). These results suggest that either one or both LTR sequences are required for *rex* regulation but that the *gag* sequence is not. The increase of CAT mRNA in parallel with CAT activity indicates that $p27^{rex}$ did not affect translation efficiency.

To determine which LTR is required for the regulation, the 3′ LTR of pL-CAT-L was replaced by the transcriptional terminator of the TK gene of herpes simplex virus 1 (31) to construct pL-CAT-TK (Fig. 2*A*). CAT expression by pL-CAT-TK was not activated by $p27^{rex}$ (Fig. 2*B*), demonstrating that the 3′ LTR is required for *rex* regulation. In the absence of $p27^{rex}$, the CAT activity expressed by pL-CAT-L was reproducibly lower than that expressed by pL-CAT-TK, which suggests a moderate suppressive effect of the 3′ LTR on CAT expression. However, activation of pL-CAT-L by $p27^{rex}$ was much greater than simple restoration of the original activity (Fig. 2*B*). A similar requirement of the 3′ LTR for *rex* regulation was also confirmed with the defective proviral construction pGAGdm (data not shown). Thus, the 3′ LTR was essential for *gag* expression.

Finally, the 5′ LTR of pL-CAT-L and of pL-CAT-TK was replaced by the simian virus 40 early promoter to construct pSV-CAT-L and pSV-CAT-TK, respectively. $p27^{rex}$ did not affect the CAT expression of pSV-CAT-TK but slightly activated that of pSV-CAT-L (Fig. 2*B*). Thus, we concluded that both the 5′ and the 3′ LTR were required for the $p27^{rex}$-mediated accumulation of mRNA that resulted in high expression of the CAT or *gag* gene, although the 3′ LTR alone conferred a partial response (Fig. 2*B*).

It was possible, however, that activation of *gag* or CAT gene expression by $p27^{rex}$ might be mediated by enhancement of *tax* expression, since a *tax* expression plasmid was always present and expression of the CAT constructions was $p40^{tax}$-dependent. However, this possibility was excluded by two observations: (*i*) the level of $p40^{tax}$ was not increased by $p27^{rex}$, as determined by protein blot analysis (data not shown; see ref. 28), and (*ii*) $p27^{rex}$ did not further activate pL-CAT-TK, the expression of which is $p40^{tax}$-dependent. In addition to activation with $p27^{rex}$, some inhibition (20–40%) of pL-CAT-TK expression was observed reproducibly at higher doses of *rex* expression plasmid (Fig. 2*B*; see also Fig. 4). The reason for this inhibitory effect is unknown, but the effect seems not to be specific to a particular promoter or enhancer and appears to be due to a high level of $p27^{rex}$.

Essential Sequence in the 3′ LTR. To identify which sequences in the 3′ LTR are responsible for regulation, we examined the effects of deletion mutations; the TK termina-

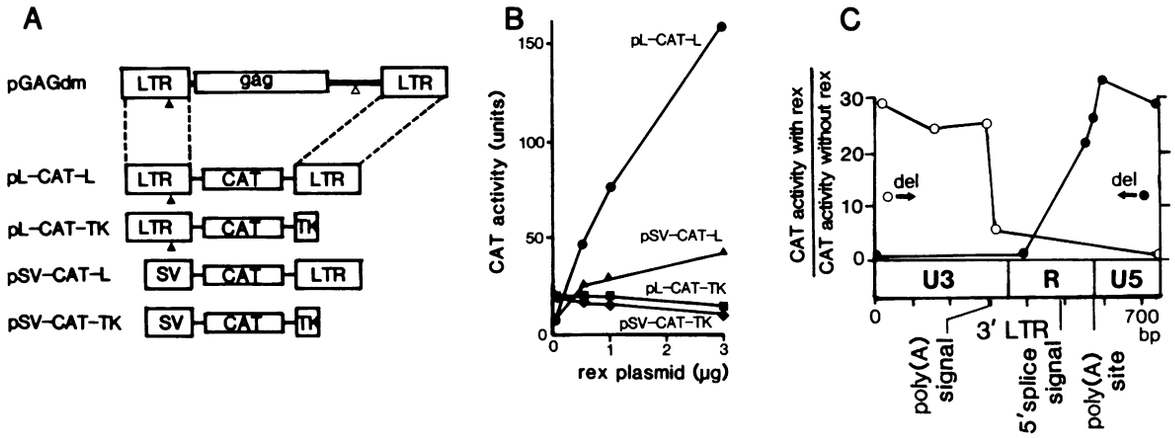

FIG. 2. Identification of regions required for *rex* regulation. (*A*) Substitution constructions of defective provirus clone pGAGdm. Boxes represent LTR, sequence from map position (m.p.) 31 to 755; CAT, fragment between the *Hin*dIII and *Sau*3AI sites of plasmid pSV2cat; SV, *Pvu* II–*Hin*dIII fragment of the simian virus 40 early promoter; TK, *Sma* I–*Pvu* II fragment of the herpes thymidine kinase (TK) gene containing the transcriptional terminator. (*B*) Effect of $p27^{rex}$ on CAT gene expression with various constructions. With each 1 μg of CAT construct and 1 μg of *tax* expression plasmid, the indicated amount of *rex* expression plasmid was cotransfected. A unit of CAT activity was defined as 1% acetylation of chloramphenicol per 30 μg of protein per 60 min. (*C*) Element in the 3′ LTR responsible for *rex* regulation. The 3′ LTR of pL-CAT-L was deleted from either terminus by using exonuclease BAL-31, and the TK terminator was inserted downstream of the 3′ LTR. CAT activities were analyzed after cotransfection of 1 μg of CAT plasmid and 1 μg of *tax* plasmid in the presence of 3 μg of *rex* plasmid or in the absence of *rex* plasmid. Activation of CAT expression by $p27^{rex}$ is defined as the ratio of CAT activity in the presence of $p27^{rex}$ to that in the absence of $p27^{rex}$. The activation with each mutant is plotted against the site of deletion in the LTR. Open circles represent deletions (m.p. 30, 154, 302, 319, and 755, respectively) from the 5′ end and solid circles represent deletions (m.p. 755, 600, 585, 560, 395, and 30, respectively) from the 3′ end. U3 and U5, unique sequences at the 3′ and 5′ termini of HTLV-I RNA; R, terminally repeated sequence.

Table 1. Activation of CAT expression at the mRNA and protein levels by $p27^{rex}$

Transfected DNA, μg			Relative CAT expression, %	
pLCatL	*tax*	*rex*	mRNA	Enzyme
3	0	0	0.1	0.1
3	3	0	15	15
3	3	1	77	87
3	3	3	100	100

The level of CAT enzyme and mRNA with each dose of *rex* expression plasmid is expressed as a percentage of the value with 3 μg of the *rex* expression plasmid. The RNA level was estimated by densitometric scanning of an autoradiogram after RNA blot hybridization, and the value was normalized to that of actin mRNA in each sample.

tor was inserted downstream of the 3′ LTR to terminate possible readthrough transcription when the LTR had a mutation.

Serial deletions from the 3′ end of the 3′ LTR in pL-CAT-L revealed that deletion of the U5 and R sequences 20 bases upstream from the poly(A) site (m.p. 580 bp from the 5′ end of the LTR; ref. 6) did not affect the *rex*-dependent activation (Fig. 2*C*). Further deletion to m.p. 395, leaving 40 bp of the 5′ region of the R sequence, completely abolished the response to $p27^{rex}$. Thus, the 3′ boundary of the essential sequence was concluded to be located between m.p. 395 and 560. A deletion mutation from the 5′ end of the LTR to m.p. 302, eliminating the enhancer sequence, did not affect the activation, but a further deletion of 17 bp (m.p. 319) completely abolished the activation (Fig. 2*C*). Thus, the 5′ boundary of the 3′ essential element was localized between m.p. 302 and 319. Therefore, we concluded that the 3′ cis-acting element for *rex* regulation resides between m.p. 302 and 560.

To confirm that this defined sequence is sufficient for *rex* regulation and to examine its function, we tested the effects of position and orientation of this sequence. To this end, a fragment containing the m.p. 302–620 region of the LTR (dL) was inserted upstream or downstream of the TK terminator in pL-CAT-TK in both orientations (Fig. 3). pL-CAT-dLα-TK, which has the dL fragment in the sense orientation upstream of the TK terminator, responded to *rex* regulation. Therefore, it was concluded that the dL sequence is sufficient as a 3′ element. Conversely, pL-CAT-TK-dLα, in which the dL fragment was inserted downstream of the TK terminator, did not respond to *rex* regulation. Transcriptional termination

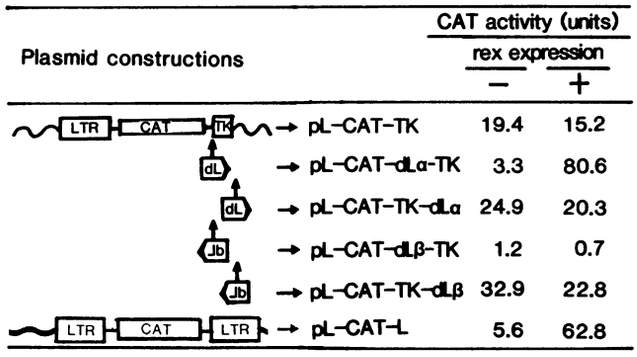

FIG. 3. Effect of position and orientation of the 3′ element. dL, a DNA fragment extending from m.p. 302 to 620 of the LTR and containing the 3′ cis-acting sequence, was inserted upstream or downstream of the TK terminator of pL-CAT-TK in the sense (α) or antisense (β) orientation. CAT activity was assayed after cotransfection of each of the resulting plasmids and *tax* plasmid in the absence (−) or presence (+) of *rex* plasmid.

at the TK terminator of pL-CAT-TK-dLα was confirmed by RNA blot analysis with TK and LTR fragments as probes (data not shown). These results indicate that the dL sequence has to be maintained in RNA molecules for the *rex* regulation to occur. This conclusion was supported by the finding that pL-CAT-dLβ-TK, containing the dL fragment in the antisense orientation, did not respond to *rex*. From these results, we conclude that transcripts containing the dL sequence at the 3′ terminus are the target molecules for $p27^{rex}$.

During the tests on various constructions, we noticed suppressive effects of the dL insertion on basal CAT expression (Figs. 2*B* and 3). The suppressive effects are not understood exactly but could be explained by differential stabilities of RNAs, because suppression was observed only when this sequence was inserted upstream of the TK terminator (Fig. 3). Thus, these variations do not affect our conclusions on *rex* regulation. The extremely low expression of pL-CAT-dLβ-TK might be due to formation of a long double-stranded stem structure between the 5′ and 3′ ends of the mRNA, since this plasmid contains both the sense and antisense sequence of the dL. With some constructions, $p27^{rex}$ induced slight suppression of CAT expression (Fig. 3), but these suppressions were weak compared with the activation by $p27^{rex}$.

Essential Element in the 5′ Region. We have reported (30) that the level of *env* mRNA from the intact viral genome is also controlled by *rex*. To analyze essential elements for regulation of *env* mRNA, we used plasmid pMTPX (21), which contains a cDNA sequence of the *env* mRNA with a deletion in the coding region (m.p. 5777–6495). The promoter is the MT gene promoter, and the transcriptional terminator is the LTR at the 3′ end. To inactivate *tax* and *rex* in pMTPX, most of the coding sequence was deleted to construct pMTPXdm (Fig. 4). For accumulation of unspliced RNA containing *env* sequence, *rex* function was essential, and the requirement for the 3′ LTR was confirmed by analyses similar to those described above (data not shown).

In the previous section, we concluded that LTRs at the 5′ and 3′ ends were required for *rex*-dependent expression of the *gag* or CAT gene, but no 5′ LTR was present in pMTPXdm, whose expression was also regulated by *rex*. To understand this difference, we analyzed the 5′ cis-acting element in the *env* cDNA construction. Deletion of the first exon derived from the 5′ LTR (data not shown) or of the 3′ half of *env* (*Xho* I–*Hpa* I restriction fragment) did not affect *rex* regulation (see del-XH in Fig. 4). On the other hand, deletion of the 5′ half of *env* (*Nco* I–*Xho* I fragment) abolished the responsiveness to *rex* (data not shown). Therefore, the 5′ element required for regulation is located within the *Nco* I–*Xho* I fragment.

The *Nco* I–*Xho* I fragment contains a 5′ splice signal at m.p. 5183. This signal might be required for *rex* regulation, because the LTR also contains a 5′ splice site. To test this possibility, we deleted a 15-bp sequence at m.p. 5171–5185 containing the 5′ splice signal from construction del-XH to form del-XH,N. This deletion of 15 bp abolished the response to *rex* regulation; that is, the level of the primary transcript was constitutive without *rex* and was not increased further by *rex* (Fig. 4). These results might be explained simply by stating that the 5′ splice signal is just for splicing. However, the 5′ splice signal seems to have an additional significance, because the 5′ splice signal was required for accumulation of the target RNA even in the absence of the 3′ splice signal, when no spliced form was detected (Fig. 2, pL-CAT-L). Therefore, it was concluded that the 5′ splice signal is required for RNA to be a target molecule for $p27^{rex}$. The 5′ splice signal seemed to destabilize the target RNA when $p27^{rex}$ was not present, so that the RNA was spliced or degraded, and this effect of the 5′ splice signal was suppressed by $p27^{rex}$. Thus, the 5′ splice signal is involved in *rex*

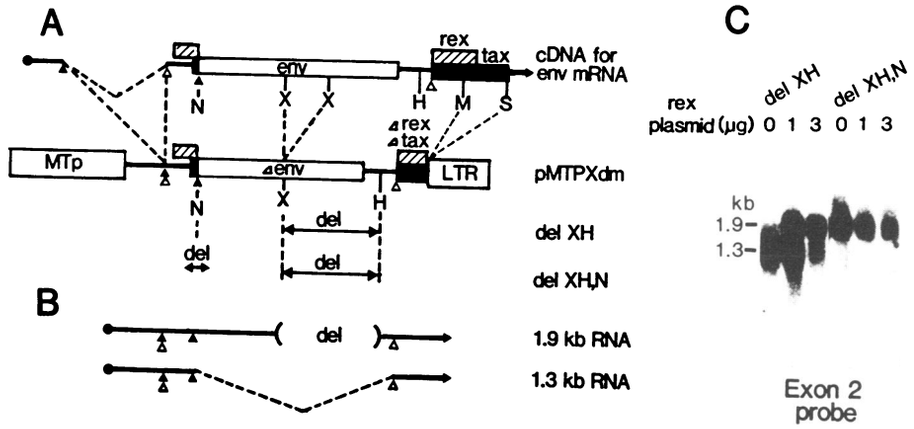

FIG. 4. Identification of the 5′ element required for *rex* regulation. (*A*) Deletion mutations in the *env* cDNA sequence in pMTPXdm (21). N, *Nco* I; X, *Xho* I; H, *Hpa* I; B, *Bgl* II; del, deletion. (*B*) RNA sizes (in kilobases, kb) expected from the constructions. (*C*) Blot analysis of RNA expressed by deletion mutants. Exon 2 hybridization probe is a *Pst* I–*Bam*HI fragment containing exon 2 of the *pX* mRNA and thus cannot detect RNA from the *rex* plasmid.

regulation. Deletion of the 5′ splice signal from the 5′ LTR of pL-CAT-L also abolished the response to *rex* regulation (data not shown).

The 5′ Splice Signal and the 3′ Element in the LTR Are Sufficient for Regulation by p27^{rex}. Cellular gene expression is probably not regulated by the *rex* gene product, because the *rex* function requires a specific sequence in the HTLV-I LTR. However, if the two cis-acting elements, the 5′ splice signal and the sequence in the 3′ LTR, are sufficient as concluded above, a cellular mRNA should become sensitive to *rex* regulation when it is linked to the LTR. To test this hypothesis, we inserted the LTR into the mouse MT-I gene (32) at the *Sac* II site in the third exon to construct pMTg-L (Fig. 5).

On transfection with pMTg-L alone, only completely spliced mRNA that was terminated at the LTR was detected. After expression of p27^{rex} in this system, two additional RNA species were detected (Fig. 5). These RNAs corresponded in size to the primary transcript (1.7 kb) and to RNA (1.2 kb) that had been spliced only at the first intron. The presence of the intron sequence in the RNA was confirmed by hybridization with the first intron as probe (Fig. 5*C*). p27^{rex}-dependent accumulation of the transcript was also observed when the LTR was inserted at the *Pst* I site in the first intron. With this construct, transcription was terminated in the LTR so that the truncated transcript had the 5′ splice signal but not the 3′ splice signal (data not shown). In contrast, the *rex* effect on RNA accumulation was no longer observed when the LTR was inserted at the *Bgl* II site in the first exon, so that the transcript had no 5′ splice signal. Therefore, we conclude that even cellular RNAs could be regulated by p27^{rex} if they had a 5′ splice signal and the target element in the 3′ LTR. These results demonstrate directly that these elements are sufficient for *rex* regulation.

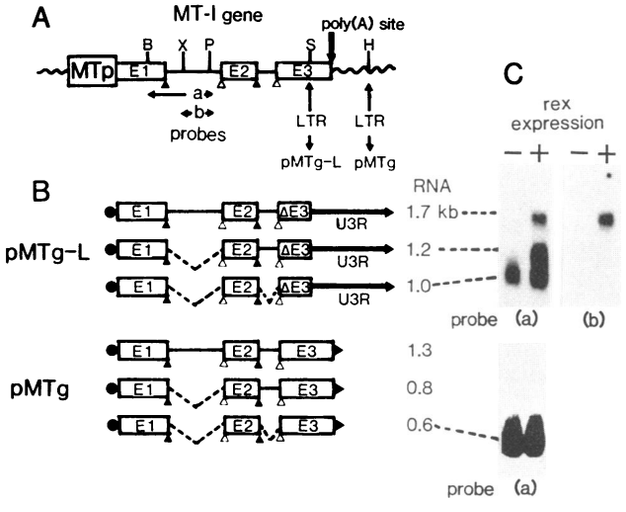

FIG. 5. *rex*-dependent regulation of a cellular gene containing the LTR. (*A*) Construction of hybrid plasmids between the MT-I gene and the HTLV-I LTR. The LTR was inserted into the *Sac* II site in the third exon (E3), to produce plasmid pMTg-L, or into the *Hin*dIII site downstream of the polyadenylylation site, to produce plasmid pMTg. Regions marked a and b indicate the hybridization probes used in *C*. E, *Eco*RI; B, *Bgl* II; X, *Xba* I; P, *Pst* I; S, *Sac* II; H, *Hin*dIII. (*B*) Sizes of RNA expected from the constructs. (*C*) Blot analysis of RNA expressed in the presence or absence of *rex* protein. The amount of RNA applied to each lane was confirmed with an actin cDNA probe (data not shown).

DISCUSSION

We previously reported that the *rex* product p27^{rex} acts to accumulate *gag* and *env* mRNAs that retain introns (29, 30). In the work described here, we identified two cis-acting elements required for this regulation: a 5′ splice signal and a specific sequence in the 3′ LTR. These two elements are sufficient for regulation by *rex*, since insertion of the LTR made a cellular gene with its own 5′ splice signal (the MT-I gene) sensitive to *rex* regulation. The target molecules for *rex* regulation are RNAs containing these two cis-acting elements—that is, p27^{rex} operates at the RNA level. A cis-acting sequence of 258 bp in the 3′ LTR contains a putative polyadenylylation signal. This may indicate that the LTR element should be located at the 3′ end of the RNA transcript.

The requirement for two elements suggests an unusual mechanism for *rex* regulation: RNA transcripts containing a 5′ splice signal are either spliced or degraded when the RNA has no 3′ splice signal, and thus they cannot accumulate in the cytoplasm. When these RNA species have a specific element derived from the 3′ LTR, p27^{rex} interacts with this element and then modulates the process. The modulation could occur by direct suppression of splicing or by acceleration of transport of the RNA into the cytoplasm: transport of nonspliced RNA could be accomplished by interfering with spliceosome formation, by opening another transport pathway, or by stabilizing the target molecules. The second possibility seems most likely, because the 3′ splice signal is dispensable for regulation by *rex*. However, further studies are required to distinguish among these possibilities.

Expression of HTLV-I genes is controlled by two trans-acting factors that regulate serial processes at different levels:

the *tax* gene product, $p40^{tax}$, activates transcription; then the *rex* gene is expressed and its product, $p27^{rex}$, modulates RNA processing, resulting in expression of *gag*- and *env*-encoded proteins. Thus $p40^{tax}$ can fully activate viral genome transcription before *rex* is expressed—that is, before high production of viral antigens. This lag time results in efficient viral production before initiation of the host immune response. On the other side, this modulation results in a reduction of the level of spliced *pX* mRNA (30). As *pX* mRNA encodes these two regulatory proteins, this reduction eventually reduces the transcriptional activation. Because of this feedback control by the *rex* gene product, HTLV-I gene expression becomes moderate or transient. The poor replication of HTLV-I and a tendency toward the latent form *in vivo* may be explained by this *rex* effect. As a result, infected cells could escape rejection by the host immune response. These situations might explain why most of the proviruses integrated in adult T-cell leukemia cells are intact (5).

Both *rex* regulation and *tax* regulation seem to be common to viruses of the HTLV subgroup, including HTLV-II (7, 8), simian T-cell leukemia virus type I (33), and bovine leukemia virus (9), since these viruses have a *pX* region that contains at least two overlapping genes. However, although other oncoretroviruses do not contain a sequence equivalent to the *pX* region, they nevertheless accumulate unspliced mRNA for expression of viral proteins. Whether other oncoretroviruses have a specific regulatory mechanism equivalent to *rex* regulation or whether they have a completely different mechanism is an open question.

Another replicative retrovirus isolated from humans, human immunodeficiency virus (HIV), has similar regulation, with two trans-acting regulatory genes, *tat* (34) and *rev* (previously termed *trs/art*) (35, 36), although they have no nucleotide sequence homologies with HTLVs. The mechanisms of these two functions of HIV seem to be different from those of the HTLV regulators (35–37, 39), although the HIV and HTLV regulatory systems are similar to each other in having overlapping genes encoding trans-acting factors that operate at different levels, recognizing signals for both activation and suppression. Therefore, the significance of *tax* and *rex* regulation discussed above might be extended to *tat* and *rev* of HIV.

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