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Post-transcriptional regulator (*rex*) of HTLV-1 initiates expression of viral structural proteins but suppresses expression of regulatory proteins

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Gene expression of human T-cell leukemia virus type 1 (HTLV-1) is regulated by two *trans*-acting factors encoded by the pX region, $p40^{tax}$ and $p27^{rex}$. $p40^{tax}$ is a transcriptional activator and $p27^{rex}$ is a post-transcriptional regulator. Using full-length viral DNA, we studied the regulatory effects of *rex* on HTLV-1 gene expression. $p27^{rex}$ is required for expression of both *gag* and *env* proteins, increasing the level of their mRNAs. The effect was dependent on the dose of $p27^{rex}$ expression plasmid. In parallel, increased doses of $p27^{rex}$ suppressed the expression of fully spliced pX mRNA, which encodes the regulatory proteins. These two effects of $p27^{rex}$ operated at the post-transcriptional level and were independent of transcriptional regulation. Lowering the level of pX mRNA down-regulates transcription of the proviral genome. These observations demonstrate that *rex* is a positive post-transcriptional regulator for *gag*, *pol* and *env* protein expression, and acts at the same time as an indirect negative regulator of viral transcription.

Key words: HTLV-1/*trans*-activation/splicing/post-transcriptional regulation

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is an exogenous human retrovirus (Poiesz *et al.*, 1980; Yoshida *et al.*, 1982). Identical geographical distribution of the virus and of adult T-cell leukemia (ATL) (Hinuma *et al.*, 1981; Kalyanaraman *et al.*, 1982), provirus integration into leukemic cells (Yoshida *et al.*, 1982, 1984; Wong-Staal *et al.*, 1983) and *trans*-activation of a cellular gene for growth-factor receptor (Inoue *et al.*, 1986a; Cross *et al.*, 1987; Maruyama *et al.*, 1987) suggest that HTLV-1 is an etiologic agent of ATL. Persistent high titers of antibodies to HTLV-1 for long periods before leukemogenesis may imply that HTLV-1 is replicating and spreading within inviduals, thus regulation of viral replication is important to understanding development of ATL.

The HTLV-1 genome contains *gag*, *pol* and *env* genes, and an extra region called 'X' or 'pX'. Three viral mRNAs, 8.5, 4.2, and 2.1 kb, are required for HTLV-1 replication. These subgenomic RNAs are formed by splicing from a single species of transcript (Seiki *et al.*, 1985a): 8.5-kb RNA is the primary transcript, and is used for expression of *gag* and *pol* proteins and also as the progeny viral genome; 4.2-kb RNA, which is formed by single-step splicing, is used for expression of *env* protein; and 2.1-kb RNA, formed by two-step splicing is used for expression of three pX proteins. These pX proteins are $p40^{tax}$ (Kiyokawa *et al.*, 1984; Lee *et al.*, 1984; Miwa *et al.*, 1984; Slamon *et al.*, 1984), $p27^{rex}$ and $p21^{X\text{-}III}$ (Kiyokawa *et al.*, 1985). Previously, we and others reported that one of the pX proteins, $p40^{tax}$ (previously termed $p40^{x}$), is a transcriptional *trans*-activator of the viral genome, thus essential for efficient transcription of viral genome (Sodroski *et al.*, 1984, 1985; Chen *et al.*, 1985; Felber *et al.*, 1985; Fujisawa *et al.*, 1985; Seiki *et al.*, 1985b, 1986).

For HTLV-1 gene expression, processing of the primary transcripts into subgenomic RNAs should be controlled, because a balance of three species of spliced and unspliced forms of mRNA are required for viral gene expression. Similar regulation is also common in retroviral replication. These requirements for accumulation of unspliced forms of mRNA are in contrast to cellular mRNA, in which all introns are rapidly spliced out. However, the mechanism of this unique regulation has been totally obscure. Recently, we (Inoue *et al.*, 1986b, 1987) have reported that the second pX protein, $p27^{rex}$ (previously termed $p27^{X\text{-}III}$), is a post-transcriptional regulator which increases unspliced mRNA levels, thus enhancing expression of *gag* protein. That is, without $p27^{rex}$, all RNA transcripts from a grossly defective provirus genome were fully spliced, and unspliced mRNA for *gag* protein was not detected. When $p27^{rex}$ was complemented, accumulation of unspliced mRNAs was induced. Therefore, it was of interest to study the effects of $p27^{rex}$ on the entire process of viral gene expression, especially focusing on the effects on expression of the *env* gene and the regulatory region pX.

Here, we report that $p27^{rex}$ function is essential for accumulation of *gag* and *env* mRNAs transcribed from the full-length proviral genome, a trigger for expression of viral structural proteins. Furthermore, $p27^{rex}$ reduces pX mRNA, which codes for regulatory proteins including $p40^{tax}$ and $p27^{rex}$, and in this way down-regulates viral gene transcription.

We will use the terms *tax* and *rex* for genes encoding $p40^{tax}$ and $p27^{rex}$, since these were suggested at a meeting on RNA tumor viruses at Cold Spring Harbor in 1987.

Results

Differential expression of subgenomic mRNA after transfection with whole viral genome

In isolated fibroblastic or epithelial cell clones expressing HTLV-1 genes, we often observed gradual suppression of viral gene expression, leading to latent state of the proviral genome (J.Inoue *et al.*, unpublished observations). These phenomena did not result from irreversible changes of the proviral structure in the transfected cells, because TPA treatment of the transfected cells could induce expression of the proviral genome. Although similar phenomena are sometimes observed with other retroviruses, we suspect that

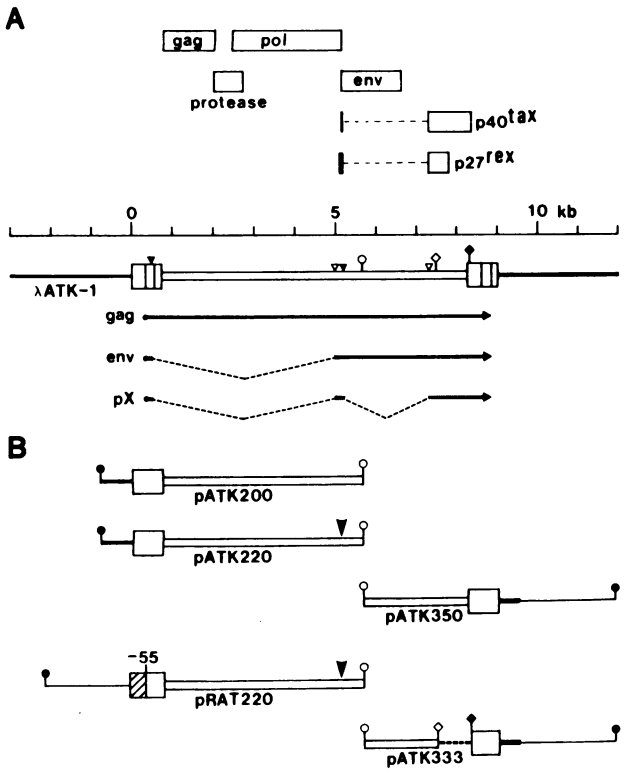

Fig. 1. Schematic illustration of HTLV-1 gene expression and construction of intact and mutant whole proviral DNAs. (**A**) Pattern of RNA splicing and coding regions is illustrated. λATK-1 (Seiki *et al.*, 1983) is proviral clone containing cellular flanking sequence (thick bar) at both ends. gag, env and pX represent the corresponding mRNAs. Closed and open triangles on the proviral genome are 5′ and 3′ splice signals; open circle stands for the *SalI* site; and open and closed squares stand for the *MluI* and *SmaI* sites, respectively. Open boxes over the proviral genome represent coding regions of protein, but do not represent the reading frames. (**B**) Strategies for construction of whole proviral DNA and its mutants. Basically, whole viral DNA was constructed by ligation of two fragments, pATK200 or its derivatives and pATK350 or its derivatives, at the *SalI* site just before transfection, then digested at the *Eco*RI sites (closed circles). Arrowheads on the proviral genome pATK220 and pRAT220 represent a single base deletion at the 5′ region of *rex* and the dotted line in pATK333 represents a deletion in the pX region. The box with oblique lines on pRAT220 represents the sequence containing RSV enhancer.

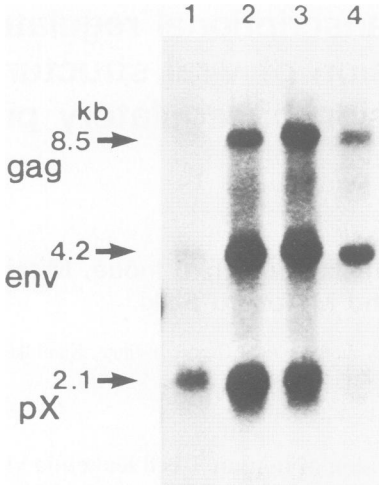

Fig. 2. Differential expression of subgenomic viral RNAs in time-dependent fashion after DNA transfection. Intact whole viral DNA (10 μg) was transfected into FL cells (6×10^5) and cytoplasmic poly(A) RNA was prepared at 10 (**lane 1**), 16 (**2**), 26 (**3**) and 52 h (**4**) after transfection and analyzed by blotting assay using a pX-specific probe.

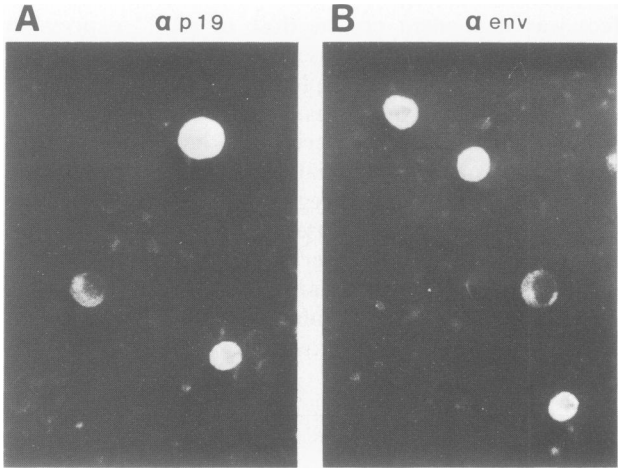

Fig. 3. Immunofluorescence staining of *gag* and *env* proteins expressed in cells transfected with full-length viral DNA. Cells transfected with the intact proviral DNA were removed from dishes by EDTA treatment after 40 h, fixed on glass slides with acetone–methanol (3:7), then incubated with mouse monoclonal antibodies against either *gag* protein p19 (**A**) or *env* gp21 (**B**). The second antibody was FITC-conjugated anti-mouse IgG antibodies.

HTLV-1 may be capable of suppressing its own gene expression. To determine the general pattern of gene expression from the intact provirus genome, we analyzed subgenomic mRNA expressed after transfection of intact proviral DNA.

The intact provirus genome was reconstructed by ligation of two fragments, pATK200 and pATK350, as summarized in Figure 1, and was transfected into a human epithelial cell line, FL cells. Cytoplasmic poly(A) RNA was isolated at various time intervals after transfection, and the subgenomic viral mRNA was analyzed by use of a blotting procedure (Figure 2). Three bands of 8.5, 4.2 and 2.1 kb RNA were detected as major components. These were identified as *gag, env* and pX mRNA respectively because of their sizes and hybridization with gene specific probes (see Figure 4).

Very interestingly, only the 2.1-kb pX mRNA species was detected at 10 h after transfection; 8.5-kb *gag* and 4.2-kb *env* mRNAs were detected only in trace amounts (Figure 2, lane 1). At 16 h, 8.5- and 4.2-kb RNAs began to appear as strong bands, and the level of 2.1-kb mRNA was also increased (lane 2). Further incubation induced a continued increase in both 8.5- and 4.2-kb RNA but a slight decrease of 2.1-kb pX mRNA (lane 3). At 52 h incubation, 8.5- and 4.2 kb RNAs could be detected as major bands, but the level of pX mRNA was drastically reduced and became difficult to detect (lane 4). The order of appearance of the subgenomic RNAs could be categorized into three stages: in the first stage, only 2.1-kb pX mRNA is present; in the second stage, all three viral mRNAs are present; and in the third stage, no or only a trace amount of 2.1-kb pX mRNA is present, but 8.5- and 4.2-kb mRNAs are observed. This process does not involve merely the appearance or disappearance of intermediates of RNA processing, since the order was reversed: 8.5-kb genomic RNA is a primary transcript and pX

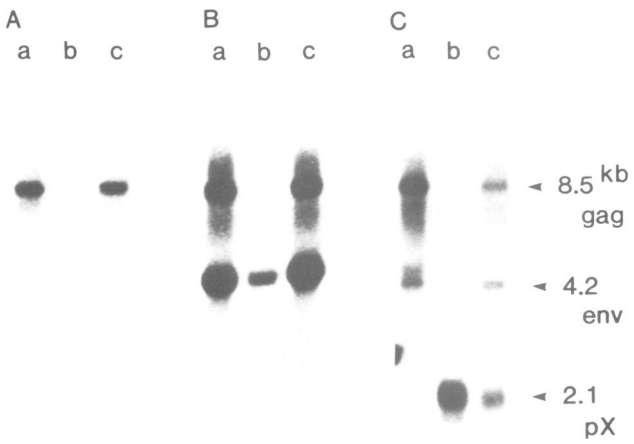

Fig. 4. Induction of *gag* and *env* mRNAs by *rex* expression. Whole proviral DNA (pATK220-pATK350, 10 μg) with one base deletion in *rex* was transfected into FL cells with (**lane c**) or without (**lane b**) *rex* expression plasmid pSRαCXsn-IV^{ter} (4 μg). Poly(A) RNA was prepared 40 h after transfection. The intact proviral DNA was similarly used as control (**lane a**) and filters were probed with *gag* (**A**), *env* (**B**) or pX sequences (**C**).

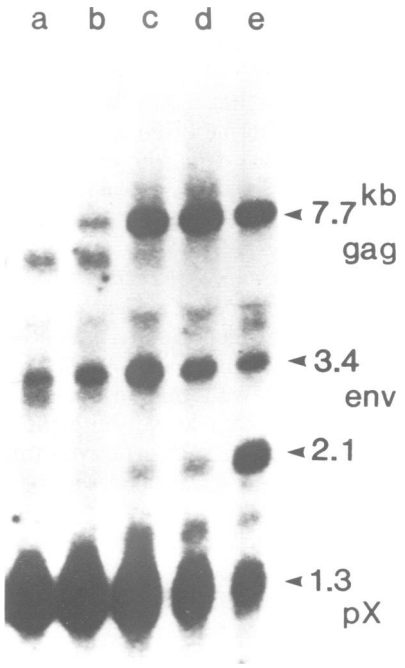

Fig. 5. *rex*-dependent induction and reduction of *gag*, *env* and pX mRNAs. Proviral DNA, pRAT220-pATK333 (10 μg), which has RSV enhancer and deletion in the pX region, was transfected into FL cells with increasing doses of *rex* expression plasmid pSRαCXsn-IV^{ter}: 0 μg (**lane a**); 0.03 μg (**b**); 0.1 μg (**c**); 0.3 μg (**d**); 1.0 μg (**e**). The RNA was analyzed 40 h after transfection using pX sequence as probe.

mRNA is a fully processed product. This finding suggests specific regulation.

At 52 h, the total levels of viral mRNAs were reduced. This cannot be explained by non-specific degradation of plasmid DNA or viral RNAs, because 2.1-kb pX mRNA was more selectively reduced. The most plausible explanation is that the reduced level of p40^{tax}, which was a consequence of reduction of pX mRNA, led to reduction of viral gene transcription.

Expression of viral proteins was confirmed in a separate experiment: *gag* and *env* proteins were detected at 40 h by indirect immunofluorescent staining with monoclonal antibodies, ATLV11 to p19 (R.Ueda, unpublished observation) and F10 to gp21 (K.Sugamura *et al.*, 1984) (Figure 3).

p27^{rex} is a trigger for the late phase of viral gene expression

Kinetic analysis of subgenomic RNA appearance suggests the existence of a specific mechanism that can switch one phase of gene expression to another. A viral product produced in an early phase might be responsible for this regulation. In this respect, the function of p27^{rex} could be relevant. We have previously reported that p27^{rex} is required for accumulation of unspliced RNA transcribed from a grossly defective provirus construction and is therefore necessary for *gag* protein expression (Inoue *et al.*, 1986b, 1987).

To test the possibility of p27^{rex} is responsible for control of differential expression of the subgenomic mRNAs, as observed in Figure 2, we used a spontaneous mutant, pATK220, which has one base deletion at the 5′ region of the *rex*, thus inducing a frame shift in the coding region of *rex* but not of *tax* (see Figure 1). The *rex* mutant DNA was transfected into FL cells and viral RNAs were analyzed by a blotting procedure. At 40 h after transfection, only 2.1-kb pX mRNA was accumulated, but no *gag* and only a low level of *env* mRNA were detected (Figure 4, lane b). The low levels of *gag* and *env* mRNA were further confirmed with gene-specific probes (Figure 4A and B). This result was similar to the early phase (10 h) of expression of the intact provirus genome, but was in sharp contrast to the late phase of gene expression in which all three viral RNAs were accumulated. Since incubation for 40 h should be long enough to shift to the late phase as confirmed with the intact DNA (lane a), this observation with *rex* mutant demonstrated that the *rex* is essential for accumulation of *gag* and *env* mRNA. No *gag* and *env* proteins were detected by immunostaining with monoclonal antibodies (data not shown).

When the mutant proviral DNA was co-transfected with a *rex* expression plasmid, expression of genomic and *env* mRNAs was induced and each mRNA was confirmed by gene-specific probes (Figure 4, lane c). Expression of both *gag* and *env* proteins was also confirmed by indirect immunostaining (data not shown). The *pol* gene product was not tested; however, it is expected to be expressed, because *pol* is translated from the same mRNA as for *gag* protein. These results clearly indicate that *rex* function is essential for accumulation of *gag* and *env* mRNAs, which still carry signals for splicing.

In these assays, *tax* was always present to maintain activated transcription. To confirm that p27^{rex} is sufficient for post-transcriptional enhancement of *gag* and *env* expression, we constructed a proviral clone whose transcription is independent of *tax* function. The transcriptional enhancer sequence, which consists of 21-bp repeats in the long terminal repeat (LTR) and is p40^{tax} dependent, was substituted with enhancer of the Rous sarcoma virus (RSV), pRAT220. The provirus construct with this modified promoter, carrying a deletion in the pX coding sequence, accumulated only spliced

RNA without $p40^{tax}$ (Figure 5, lane a). However complementation of the *rex* function alone resulted in accumulation of *gag* and *env* mRNAs (7.7- and 3.4-kb RNAs in this defective construction; Figure 5, lane b–e). These results were similar to those with the original provirus construction (Figure 4). Thus it was demonstrated that *rex* function alone is sufficient for post-transcriptional regulation that increases levels of viral RNAs carrying splicing signals.

$p27^{rex}$ down-regulates expression of pX mRNA, which codes for transcriptional activator

We observed a selective reduction of 2.1-kb mRNA at a late stage after transfection (Figure 2, lane 4). The *rex* function may also be associated with this regulation, since we previously reported that a high dose of *rex* expression DNA led to a reduction of a spliced RNA derived from grossly deleted provirus. Therefore, it was of interest to test directly the effect of *rex* on pX mRNA, which is formed by double splicing. To test this, proviral DNA with an 826-bp deletion of the pX region but directed by the RSV enhancer pRAT220-pATK333 was constructed. The size of the deletion in the pX sequence made it possible to distinguish the defective mRNA of the provirus from that expressed by the *rex* expression plasmid. The proviral DNA with the deletion mutation was co-transfected with increasing doses of *rex* expression plasmid pMTCXsn-IV^{ter}, and viral RNA expressed was analyzed 40 h later. As expected on the basis of the observations above, 7.7-kb *gag* and 3.4-kb *env* mRNAs increased in parallel with the increase of 2.1-kb *rex* mRNA; however, 1.3-kb pX RNA decreased. The 3.4-kb *env* mRNA decreased after an initial increase with higher doses of *rex*. These results clearly indicate that expression of *rex* resulted in an increase of *gag* and *env*; however, overexpression can also induce a decrease of *env* mRNA. Therefore, it was concluded that $p27^{rex}$ is also responsible for suppression of levels of spliced pX and *env* mRNAs. In this way, $p27^{rex}$ would eventually down-regulate transcription of the viral genome, because pX mRNA encodes the *trans*-activator required for efficient transcription. Some faint, unexpected bands were sometimes observed (Figure 5). However, these were not reproducible and were therefore considered to be transcripts from undesired ligation of DNA fragments because the ligation mixture had been used for transfection without further purification.

Discussion

For gene expression from the whole viral genome of HTLV-1 a *rex* product, $p27^{rex}$, is required to accumulate viral mRNAs coding for *gag, pol* and *env* proteins, whose mRNAs are not fully spliced. On the other hand, this product reduces the level of fully spliced pX mRNA coding for regulatory proteins. The reduction of pX mRNA eventually down-regulates transcription of the HTLV-1 genome. Therefore, it could be concluded that $p27^{rex}$ is a positive regulator for expression of viral structural proteins and a negative regulator for viral gene transcription.

This function of *rex* in causing the accumulation of *gag* and *env* mRNAs is of particular interest for understanding retroviral replication. In general, retroviral replication requires both spliced and unspliced forms of RNAs for mRNAs and genomic RNA. These requirements are in sharp contrast to the cellular mRNA, for which introns are very rapidly spliced out; otherwise, unspliced RNA is destroyed. However, the mechanism for specific maintenance of unspliced forms of viral mRNAs has not been understood. Since $p27^{rex}$ initiates accumulation of two species of unspliced viral RNA, *rex* confers this regulation for HTLV-1. All members of the HTLV subgroup that possess a pX region contain the second pX gene, which is equivalent to the *rex* (Sagata *et al.*, 1985). It can therefore be expected that a similar protein is involved in such regulation. However, it is still unclear whether regulation of retroviruses in general, which do not possess a pX-equivalent gene, is by means of *rex*-equivalent regulation or by a completely different mechanism.

Differential effects of *rex* on each viral RNA species were also observed. Accumulation of *gag* mRNA, which is a primary transcript, was stimulated by increasing doses of *rex*; however, *env* mRNA initially increased, then was gradually reduced. Since *env* mRNA is formed by single-step splicing of the primary transcript and is further spliced into fully spliced pX mRNA (Seiki *et al.*, 1985a), suppression of the second process will increase the *env* mRNA level but suppression of the first process will reduce the level. Therefore, independent modulation of each processing event by $p27^{rex}$ can explain the initial increase of *env* mRNA levels, followed by a decrease. The molecular mechanism of *rex* function is not yet well understood. However, our previous observations (Inoue *et al.*, 1987) have suggested that the splicing process itself is not a direct target of *rex* function, because construction wihtout the 3′ splicing signal was also controlled by *rex*. As a mechanism of this regulation, stabilization or acceleration of transport of precursor RNAs has been postulated.

The *rex* induced an increase of *gag* and *env* mRNAs, but at the same time a decrease of pX mRNA in a dose-dependent fashion. Reduction of the pX mRNA level would naturally result in reduction of $p40^{tax}$ which is a *trans*-activator of LTR, and thus would therefore eventually suppress transcription of the viral genome. This conclusion is also supported by the fact that formation of pX mRNA from the LTR-directed proviral DNA was more affected by the *rex* than that with RSV enhancer-directed provirus, whose expression was not affected by $p40^{tax}$ (compare Figures 2 and 5). Therefore, well-balanced expression would be important for efficient expression of viral genes and replication. In this respect, it is noteworthy that both *tax* and *rex* are expressed by a single mRNA. However, it is possible that differences in the stability of $p40^{tax}$ and $p27^{rex}$ or alterations in their translation efficiency may result in unbalanced expression of $p40^{tax}$ and $p27^{rex}$. Although unbalanced expression was not directly demonstrated, it would result in suppression of viral gene expression. Poor replication competence and a tendency towards the latent form (Clarke *et al.*, 1984; Kitamura *et al.*, 1985; Kiyokawa *et al.*, 1985) may be explained in part by possible unbalanced expression of *rex* and *tax*. In fact, primary leukemic cells are not expressing viral antigen and this would explain why the intact proviral genomes are maintained in primary leukemic cells in most cases (Yoshida *et al.*, 1984). This situation is in contrast to avian proviruses in chicken tumors induced by viral infection, in which defective genomes are usually maintained. If an intact genome expresses the viral proteins efficiently, transformed cells would be rejected by the host's immune

response to viral proteins (Hayward *et al.*, 1981).

On the basis of the *rex* function required for expression of viral structural proteins, HTLV-1 gene expression can be separated into two phases: early and late. In the early phase, all or most viral transcripts are spliced into pX mRNA which expresses *tax* and *rex*, and the *tax* further activates the transcription. When an early gene product *rex* protein accumulates to a certain level, it induces expression of *gag*, *pol* and *env* proteins. In this scheme, *rex* is an early gene and its product is a trigger for the late phase of gene expression. The idea of two phases in HTLV-1 replication is similar to the situation with DNA tumor viruses, in which transcriptional activators are triggers for the late phase. The significance of triggering the late phase is similar for both DNA viruses and HTLV-1, but the mode of function of the *rex* is unique.

Two regulatory genes, *tat* and *art/trs*, have also been proposed for the human immunodeficiency virus (HIV) (Sodroski *et al.*, 1985, 1986; Feinberg *et al.*, 1986). These appear to be similar to some extent with respect to overlapping genes and transcriptional and post-transcriptional regulators respectively. Since HTLV and HIV belong to different groups of the retroviruses and have different genomic constitutions, and also since no significant homologies of their nucleotide sequences have been detected, it would be of interest to study the similarities and differences, the origin and the significance of these two regulatory genes.

Materials and methods

Plasmid construction

A DNA clone (λATK-1) (Seiki *et al.*, 1983) with a full length of the HTLV-1 genome was separated into two fragments at the *Sal*I site (5672 bp from the 5′ end of the provirus) and maintained in pBR322 separately because the full-length clone was unstable in *Escherichia coli*. The 5′ portion, pATK200, contained a 5′ cellular flanking sequence, LTR, *gag*, *pol* and the 5′ half of *env* sequences; the 3′ portion, pATK350, contained the 3′ half of *env*, pX, LTR and 3′ cellular flanking sequences.

Plasmid pATK220 is a mutant of pATK200 and has a single base deletion at 5170 bp, which is in the *rex* coding region but upstream of *tax*, thus inactivating *rex* but not *tax* expression. Plasmid ATK333 is a mutant of pATK350 with an 826-bp deletion in the pX region which was excised by *Mlu*I and *Sma*I. Plasmid pRAT220 is a derivative of pATK220, whose enhancer in the LTR is substituted with RSV enhancer sequence: a 428-bp fragment containing RSV enhancer was cut out with *Pvu*II and *Eco*RI from RSV LTR and replaced with a sequence upstream of the *Nde*I site in the LTR, which is located at −55 bp from the cap site.

The DNA fragment with cellular flanking and the provirus sequences was cut out from each plasmid with *Eco*RI and *Sal*I, and a pair of 5′ and 3′ portions were ligated. Then the ligated mixture was digested with *Eco*RI to generate one unit of full-length provirus genome with cellular flanking sequences at both ends. The reconstructed whole viral DNA was directly used for the transfection assay.

The expression plasmid for *rex*, pSRαCXsn-IV^{ter} was described previously (Inoue *et al.*, 1987).

Transfection and RNA analysis

A human epithelial cell line, FL cells (6×10^5), was seeded in a 60-mm dish. On the next day the cells were transfected with the proviral DNA (10 μg) with or without *rex* expression plasmid (4 μg each) by the calcium phosphate method as described previously (Fujisawa *et al.*, 1985). After 40 h, unless otherwise specified, cells were harvested and cytoplasmic RNA was extracted with vanadyl complexes according to the procedure of Berger and Birkenmeier (1979). RNA containing poly(A) was isolated by oligo(dT)−cellulose and analyzed by a blotting procedure after denaturation with 2.2 M formaldehyde. The probes used were *gag* sequence (851−1370 bp), *env* sequence (5777−6495 bp), pX sequence (7268−8029 bp) or exon 1 (254−611 bp). Hybridization was carried out overnight at 42°C in a buffer containing 50% formamide and 4 × SSC. The filter was finally washed in 0.5 × SSC for 15 min at 65°C.

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