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Identification of a sequence element immediately upstream of the polypurine tract that is essential for replication of simian immunodeficiency virus

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A short stretch of T-rich sequences immediately upstream of the polypurine tract (PPT) is highly conserved in the proviral genomes of human and simian immunodeficiency viruses (HIV and SIV). To investigate whether this 'U-box' influences SIVmac239 replication, we analyzed the properties of mutants with changes in this region of the viral genome. All mutants were either retarded in their growth (up to one month delay) or did not replicate detectably in CEMx174 cells. When U-box mutants did replicate detectably, compensatory changes were consistently observed in the viral genome. The most common compensatory change was the acquisition of thymidines immediately upstream of the PPT, but marked expansion in the length of the PPT was also observed. U-box mutants produced transiently by transfection were severely impaired in their ability to produce reverse transcripts in infectivity assays. Analysis of transiently produced mutant virus revealed no defect in RNA packaging or virus assembly. These results identify a new structural element important for an early step in the viral life cycle that includes reverse transcription.

Keywords: HIV/replication/reverse transcription/SIV/U-box

Introduction

Retroviruses are single-stranded positive-sense RNA viruses which replicate via a DNA intermediate. The double-stranded DNA intermediate is synthesized from the RNA template by the viral-encoded polymerase during reverse transcription. The double-stranded viral DNA is subsequently integrated into the cellular genome where it directs viral transcription, protein expression and virus production. A number of *cis*-acting sequences are known to be critical for different steps of this replicative cycle. These include: transcription promoter and enhancer sequences located within the long terminal repeats (LTRs) of proviral DNA; the primer binding site (PBS) in RNA; RNA dimerization and encapsidation signals; and the polypurine tract (PPT) (for review see Coffin, 1990). Retroviral LTRs, generated during reverse transcription, consist of U3, R and U5 regions (Coffin, 1990).

The PPT is located immediately upstream of the U3 region, toward the 3′-end of viral RNA. It is a short, conserved, purine-rich sequence, the integrity of which is essential for retrovirus replication (Sorge and Hughes, 1982; Omer *et al*., 1984; Resnick *et al*., 1984; Smith *et al*., 1984a,b). The PPT is believed to serve as a main primer for the synthesis of plus-strand DNA during reverse transcription. This event does not take place until the early stages of reverse transcription are completed i.e. not until minus-strand DNA (initiated by $tRNA_3^{Lys}$ annealing to the PBS) is extended past the U3 region of the RNA template. During this process, the template RNA, which is part of a newly formed DNA–RNA duplex, is being efficiently degraded by RNase H activity within the same reverse transcriptase (RT) molecule which directs DNA synthesis. However, the PPT sequence is specifically protected from the RNase H activity of the RT. It is believed that this enables the short stretch of purines to serve as a primer for the synthesis of a plus strand of DNA using the previously synthesized minus-strand DNA as a template (Sorge and Hughes, 1982; Finston and Champoux, 1984; Omer *et al*., 1984; Resnick *et al*., 1984; Smith *et al*., 1984a,b; Repaske *et al*., 1989; Huber and Richardson, 1990; Luo *et al*., 1990; Pullen and Champoux, 1990; Charneau *et al*., 1992; Heyman *et al*., 1995; Lauermann *et al*., 1995; for reviews see Coffin, 1990; Champoux, 1993; Telesnitsky and Goff, 1993). The specificity of PPT excision and the role of its various nucleotides in directing the proper 3′-cut and subsequent DNA synthesis initiation have been convincingly demonstrated in a number of retroviral systems (Finston and Champoux, 1984; Smith *et al*., 1984b; Repaske *et al*., 1989; Huber and Richardson, 1990; Luo *et al*., 1990; Pullen and Champoux, 1990; Wöhrl and Moelling, 1990; Gopalakrishnan *et al*., 1992; Pullen *et al*., 1992, 1993; DeStefano *et al*., 1993; Hottiger *et al*., 1994; DeStefano, 1995; Fuentes *et al*., 1995; Lauermann *et al*., 1995; Palaniappan *et al*., 1996; Powell and Levin, 1996; Wilhelm *et al*., 1997). However, neither the mechanism of PPT protection from the seemingly non-specific degradation by RNase H nor the requirements for its 5′-end cut have been established (Oyama *et al*., 1989; Huber and Richardson, 1990; Luo *et al*., 1990; Pullen and Champoux, 1990; Wöhrl and Moelling, 1990; Pullen *et al*., 1992, 1993; DeStefano *et al*., 1993; Fuentes *et al*., 1995; Lauermann *et al*., 1995; Hughes *et al*., 1996; Palaniappan *et al*., 1996; Powell and Levin, 1996; for review see Champoux, 1993).

While comparing several SIV and HIV sequences, we noted a uridine-rich sequence located immediately upstream of the PPT. Powell and Levin (1996) have previously noted this sequence in various strains of HIV-1. We have found a similar U-rich sequence in the vast majority of other retroviruses and retroviral elements. Here we demonstrate the importance of this sequence element for an early step in the life cycle of SIV that includes reverse transcription.

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Table I. Polypurine tracts of human and simian lentiviruses

Virus	PPT 3′-LTR
SIVmac239	CATGTCTCA**TTTTAT**AAAAGAAAAGGGGGGAC∧TGGAAGGGATTT
HIV-1 (NL43)	TCTTAGCCAC**TTTTT**AAAAGAAAAGGGGGGAC∧TGGAAGGGCTAA
HIV-1 (HXB2)	TCTTAGCCAC**TTTTT**AAAAGAAAAGGGGGGAC∧TGGAAGGGCTAA
HIV-1 (LAI)	TCTTAGCCAC**TTTTT**AAAAGAAAAGGGGGGAC∧TGGAAGGGCTAA
HIV-1 (SF2)	TATTAGCCAC**TTTTT**AAAAGAAAAGGGGGGAC∧TGGAAGGGCTAA
HIV-2 (BEN)	TATGTCACA**TTTTAT**AAAAGAAAAAGGGGGAC∧TGCAAGGGATGT
HIV-2 (NIHZ)	TATGTCTCA**TTTTAT**AAAAGAAAAAGGGGGAC∧TGGAAGGGCTGT
HIV-2 (ROD)	TATGTCACA**TTTAAT**AAAAACAAGGGGGGGAC∧TGGAAGGGATGT
SIV (MND)	CTTCTCTCA**TTTTAT**AAAAGAAAAGGGGGGAC∧TGGGAGGGATAT
SIV (AGM)	TTTTTCGCAC**TTTTT**AAAAGAAAAGGGAGGAC∧TGGATGGGATTT

Thymidine-rich sequences immediately upstream of the PPT are shown in bold. PPT sequences are underlined. ∧ signifies the boundary between PPT and 3′-LTR. All sequence data are from DDBJ/EMBL/GenBank.

Table II. Polypurine tracts (PPTs) and possible U-boxes of various retroviruses and retroviral elements

Retrovirus	Genus	PPT	U-box
CAEV	lenti	CTTGGAGAACACCACAAAAATAAAAAAAGAAAGGG∧TGACTGTGAGA	no (?)
EIAV	lenti	CCTCAG**TATGTTT**AGAAAAACAAGGGGGGAAC∧TGTGGGGTTTTT	yes
FIV	lenti	TTCTGAAGATGAACC**TCCT**AAAAAAGAAAAAAGGG∧TGGACTGGGATG	yes (?)
BIV	lenti	TCC**TATTTT**AAAC**TT**AAAAGGGTGGAC∧TGTGGGGCAGGGTG	yes
SIVmac239	lenti	CATGTCTCA**TTTTAT**AAAAGAAAAGGGGGGAC∧TGGAAGGGATTT	yes
HIV-1(NL43)	lenti	TCTTAGCCAC**TTTTT**AAAAGAAAAGGGGGGAC∧TGGAAGGGCTAA	yes
Mo-MuLV	onco, type C	**TTTATTTAGTCT**CCACAAAAAGGGGGGAA∧TGAAAGACCC	yes
FeLV	onco, type C	TTCCA**TTTAGT**CCCTAGAAGAAGGGGGGAAA∧TGAAAGACCC	yes
Fr-MuLV	onco, type C	TAAAAGA**TTTTATTTAGTTT**CCAGAAAAAGGGGGGAA∧TGAAAGACCCC	yes
AMV	onco, type C	AGC**TTTTGCAT**AGGGAGGGGGAAA∧TGTAGTCTTA	yes
RSV	onco, type C	TACGC**TTTTGCAT**AGGGAGGGGGAAA∧TGTAGTCTTA	yes
BLV	onco, type C	TCTCCCGCC**TTTTTT**GAGGGGGAGTCATT∧TGTATGAAAGATC	yes
HERV-K10	onco, endo	TGGCGG**TTTTGT**CGAAAAGAAAAGGGGGAAA∧TGTGGGGAAAAGCAA	yes
MPMV	onco, type D	TGTGTC**TTTTGTTTTTTATAATT**AAAAAGGGTGACA∧TGTCCGGAGCCG	yes
MMTV	onco, type B	TCTTAACGTGC**TTCTTTT**AAAAAAGAAAAAAGGGGGAAA∧TGCCGCGCCTGC	yes
MuEV	onco, type A	**TTTGCCTCTTATT**AAAAGGAAAGGGGGAGA∧TGTTGGGAGCC	yes
Ty1	retrotransposon	CA**TTAT**GGGTGGTA∧TGTT	yes

Proviral sequences shown are located immediately upstream and downstream from the junction between PPT and 3′-LTR. PPT sequences are underlined. Thymidine-rich stretches (U-boxes) at the 5′-end of the PPTs are shown in bold. All sequences are from DDBJ/EMBL/GenBank. ∧ signifies the start of 3′-LTR. (?) signifies uncertainty regarding the presence of U-box-like sequence.

Results

Conservation of U-box sequence

Alignment of the sequences of various HIVs and SIVs revealed a striking similarity not only within the PPT itself, but also in a short stretch of nucleotides immediately upstream of it (Table I). Further sequence analysis of various representatives of the Retroviridae family revealed that the majority possess such a U-rich region (U-box) immediately upstream of the 5′-end of the PPT (Table II). Such sequences can also be seen in the yeast retrotransposons Ty1 (Table II) and Ty4. The second PPT sequence, present in the majority of lentiviruses and located within the integrase gene (Charneau *et al*., 1992), is preceded by a U-box, as is the newly-identified central PPT sequence in the genetically distant element Ty1 (Heyman *et al*., 1995; Wilhelm *et al*., 1997; data not shown). This element, which in provirus contains mainly thymidines (in the viral RNA genome these would be uridines), is thus quite conserved among all retroviruses. Caprine arthritis-encephalitis virus (CAEV) is the only exception we noted to this general conservation (Table II).

Integrity of the U-box is essential for efficient SIV replication

To investigate whether changes in the U-box affect viral replication, we prepared a collection of SIVmac239 mutants and analyzed their replicative capacities. The structure of these mutations and a brief description of the ability of mutants to replicate upon transfection into CEMx174 cells are summarized in Table III.

Replication of SIVΔU-box was usually delayed 5–10 days in comparison with the parental sequence (Figure 1A and B), SIV Ubox-sub1 and SIV Ubox-sub2 were suppressed to an undetectable level of replication (Figure 1A and C, respectively), and SIV Ubox-sub3 replicated similar to wild-type (Figure 1C). In two out of three experiments, replication of the Ubox-sub4 strain was completely inhibited (data not shown). However, in two separate single transfection experiments, Ubox-sub1 (Figure 1B) and Ubox-sub4 (Figure 1C) began to grow and reached wild-type levels of expression after a lengthy delay. Thus, viruses deficient in thymidine residues immediately upstream of the PPT, particularly at positions

Table III. Replication capacities of SIV strains with mutations in the region immediately upstream of the polypurine tract upon the transfection in CEMx174 cells

Virus strain	Sequence	Replication in CEMx174 transfection
	9432 9444	
SIVmac239	TGTCTCA**TTTTAT**ppt	++++
SIV/Ubox-sub1	TGTCTCCCCCCGCppt	–(1/7) grossly delayed (>3 weeks)
SIV/Ubox-sub2	TGTCTCATCCCGCppt	–(0/2)
SIV/Ubox-sub3	TGTCTCCCCCTATppt	++++ (2/2)
SIV/Ubox-sub4	TGTCTCAGACGTCppt	+/–(1/3) delayed >2 weeks
SIVΔUbox	TGTCTCC------ppt	+++ (4/4) delayed 5–10 days
SIVΔUbox-sub1	TGGCCCC------ppt	–(0/2)

Shown are: U-box sequence (in bold) and polypurine tract (ppt, bases 9445–9460). Introduced mutations are underlined. The number of transfections resulting in viral growth (of the total number of transfections for each strain) is shown in parentheses.

one and/or three, were significantly limited in their ability to replicate. However, wild-type or near wild-type growth kinetics were observed when the TAT sequence was preserved in those positions immediately preceding the PPT (mutant Ubox-sub3, Figure 1C). Interestingly, the SIV strain from which thymidines had been deleted (ΔUbox) grew consistently, but with considerably delayed kinetics (Figure 1A and B). However when two additional thymidines, moved closer to the PPT by deletion of the U-box, were also mutated (Ubox-sub1, Figure 1C), viral replication was once again irreversibly impeded.

SIV U-box mutants which replicate in vitro acquire reversional or compensatory changes in the U-box or PPT

Next we investigated whether reversional or compensatory mutations contribute to replication in cases where only a minority of transfections yielded replicating virus, and in cases where viral replication was consistently delayed.

Stocks of SIV derived from CEMx174 cell transfections were used for the subsequent *in vitro* infection of CEMx174 cells and primary Rhesus monkey peripheral blood mononuclear cell (PBMC) cultures (Figure 2). All viruses that consistently demonstrated delayed growth in transfection experiments also exhibited delayed post-infection replication. However, the two late-breaking strains, Ubox-sub1 and Ubox-sub4, exhibited significantly shorter delays (Figure 2A; Table IV). At the same time, replication of SIV/Ubox-sub1 was still impaired in primary Rhesus PBMC cultures (Figure 2B). The improved growth kinetics of Ubox-sub1 and Ubox-sub4 in CEMx174 cells suggested that these replication-competent strains may have acquired reversional and/or compensatory changes in the U-box region. Sequencing of proviral DNA from both transfected and infected cultures was used to provide evidence for this. Both of these replicating strains had acquired identical novel mutations resulting in the appearance of two thymidines immediately upstream of the PPT (Tables V and VI). The resulting sequence was different from the SIVmac239 original but similar to those present in most HIV-1s and SIV_{AGM} (Table I).

Strain SIVΔUbox exhibited delayed replication in infection experiments to an extent similar to that observed in transfections (Figure 2A; Table IV). It was also delayed in PBMC cultures (Figure 2B). Nonetheless, U-box or PPT structures derived from this mutant displayed significant sequence changes after first or second passage *in vitro* (Tables VII and VIII). In two independent transfections (experiments 2 and 3, Table VII, lines 4–12) we observed a variety of point mutations and additional deletions. Their patterns were similar in that they brought thymidines close to the 5′-end of the PPT either by C→T transition or by deleting short sequences located upstream of the PPT. Another ΔUbox strain (experiment 1, Table VII, line 3; Table VIII) acquired additional changes after second passage *in vitro*. In two independent infection experiments we observed the introduction of additional adenines to the PPT of this strain. This resulted in enlargement of the PPT by eight to 19 bases. In some clones this novel poly(A) sequence was 28 bases long (Table VIII).

SIVmac239 U-box mutants are defective in reverse transcription

To investigate the nature of the block to replication of U-box mutants, we examined early events in the viral life cycle that included reverse transcription. As these mutants either did not grow in lymphoid cell lines or acquired additional changes to the U-box/PPT region, viral stocks for these experiments were produced via transient expression in COS-1 cells. When transfections were performed with equal amounts of mutant and parental DNA, virus yields did not differ significantly in the concentration of major capsid protein p27 (data not shown). These stocks were used for infection of CEMx174 cells, in which the synthesis of DNA reverse transcripts and the kinetics of viral replication could be measured simultaneously (Figures 3 and 4). As shown in Figure 3, the growth curves of COS-derived virus in CEMx174 cells were essentially identical to those observed earlier from the transfection of CEMx174 cells with viral plasmid DNA (Figure 1). Mutant ΔUbox growth was delayed and mutant Ubox-sub1 did not grow at all. A mutant lacking the PPT was also replication incompetent (Figure 3).

The efficiency of viral DNA synthesis by various SIV strains was assessed by quantitative PCR amplification of early, extended and late products of reverse transcription (Figure 4A, B, C and D). The positions of primers used in amplification are shown in Figure 4F. Twenty hours post-infection the appearance of the late reverse transcription product was severely suppressed for all U-box and PPT mutants (Figure 4D). The accumulation of early reverse transcripts (strong-stop DNA) was not affected by any of the mutations analyzed (Figure 4A), nor was the accumulation of transcripts extended over the R region (Figure 4B). However, while extension of reverse transcripts through the 5′-end of the U3 region was suppressed

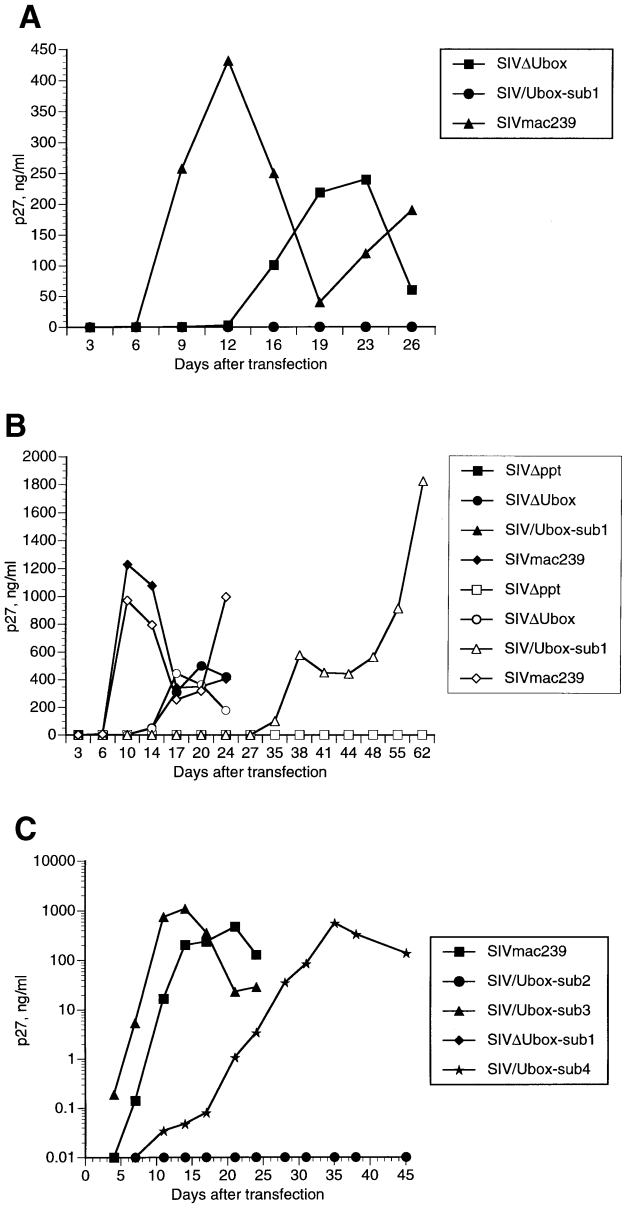


Fig. 1. Replication of SIVmac239 U-box and PPT mutants upon transfection into CEMx174 cells. (**A**) Results of transfection with SIVΔUbox and SIV/Ubox-sub1. (**B**) Results of two simultaneous independent transfections (open and closed symbols, correspondingly) with SIVΔUbox, SIV/Ubox-sub1, SIVΔppt and SIVmac239. (**C**) Results of transfection with SIV/Ubox-sub2, SIV/Ubox-sub3, SIV/Ubox-sub4 and SIVΔUbox-sub1. Results shown in Figure 1B and C represent the only experiments in which single strains of SIV/Ubox-sub1 and SIV/Ubox-sub4, respectively, have replicated after transfection of proviral DNA into CEMx174 cells.

by U-box mutations, it was not affected by the Δppt mutation (Figure 4C). This experiment was repeated several times using DNA samples from two independent sets of infections and similar results were obtained each time.

SIVmac239 U-box mutants are not impaired in RNA encapsidation and particle production

The ability of U-box mutants to encapsidate RNA and to assemble into particles was investigated. COS-1 cells were transfected with the proviral DNA of strains SIVmac239, SIV/Ubox-sub1 and SIVΔUbox, and labeled with

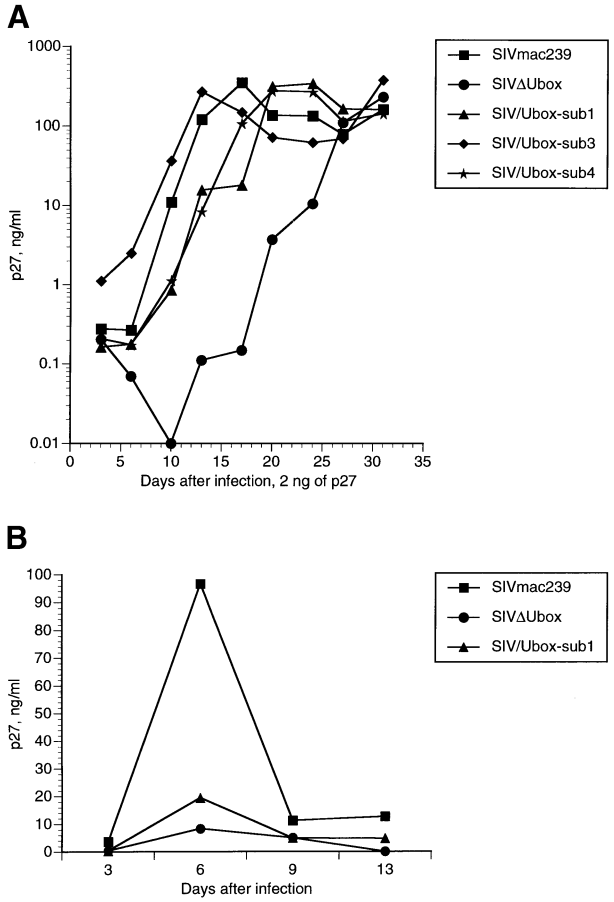


Fig. 2. Replication of SIVmac239 U-box mutants after infection of lymphoid cells *in vitro*. Virus stocks derived from transfections described in Figure 1 were used for infection of CEMx174 cells (**A**) or Rhesus monkey PBMCs (**B**).

[^{3}H]uridine. Virus-containing supernatants were then collected, concentrated and centrifuged through a sucrose gradient. Incorporated [^{3}H]uridine was measured in the collected fractions in parallel with determination of the quantity of major viral capsid protein p27. The results of these experiments are presented in Figure 5. Overall, there was no major difference between the levels of virus-associated [^{3}H]uridine in U-box mutants versus the parental SIVmac239. The peak of this incorporation was in all cases consistently observed in the area of density typical for retroviruses, and coincided with the peak of p27 (Figure 5). The average RNA:protein ratio did not differ significantly between wild-type and Ubox mutants in three independent experiments with SIVΔUbox, and six experiments with SIV/Ubox-sub1 (data not shown). Also, [^{3}H]uridine incorporation was specifically suppressed by the RNA polymerase inhibitor α-amanitin (data not shown).

Discussion

Reverse transcription is a complicated multi-step process which requires proper co-ordination of a number of *cis*-acting elements located within the retrovirus genome (reviewed by Coffin, 1990). It is initiated by reverse transcriptase which utilizes cell-derived tRNA as a primer for the synthesis of minus-strand strong-stop DNA. The

Table IV. Summary of replicative capacities and reversional events observed upon the infection of CEMx174 cells with SIVmac239 U-box mutants

Virus strain	Replication in CEMx174 infection	Type of U-box sequence recovered from infected cells; its relationship to original mutation
SIVmac239	wild-type	wild-type
SIV/Ubox-sub1[a]	delayed 3–4 days, reaches wild-type level	revertant, homogenous; acquires two thymidines at the 5′ end of PPT
SIV/Ubox- sub4[a]	delayed 3–4 days, reaches wild-type level	revertant, slightly heterogeneous; steadily accumulates two thymidines at the 5′ end of PPT
SIVΔUbox	delayed 5–10 days, reaches wild-type level	revertant, very heterogeneous; either acquires thymidines close to the 5′ end of PPT or expands PPT by incorporating additional adenines

[a]Virus stock was obtained from a single successful transfection (see Table III and Figure 1).

Table V. Nucleotide changes in SIV/U-box-sub1 proviral sequences recovered from CEMx174 cells

Virus strain	Origin of sequence	Sequence of U-box
SIVmac239	wild-type	TGTCTCATTTTATppt
SIV/Ubox-sub1	constructed	TGTCTCCCCCCGCppt
SIV/Ubox-sub1-tr (6/6)	recovered, 35 days post-transfection	TGTCTCCCCCC**TT**ppt
SIV/Ubox-sub1-inf (5/5)	recovered, 20 days post-infection	TGTCTCCCCCC**TT**ppt
HIV-1 NL-43	DDBJ/EMBL/GeneBank	TTAGCCACTTTTTppt[a]

[a]The polypurine tract sequence in HIV NL-43 and SIVmac239 is identical.
Mutant sequence is underlined. Reversions are shown in bold. Number of clones derived from transfected and infected cells and subsequently sequenced is shown in parentheses. Viral stock prepared 38 days post-transfection was used for infection.

Table VI. Nucleotide changes in SIV/Ubox-sub4 proviral sequences

Virus	Sequence	Number of clones		
		Day 31 post-transfection	Day 45 post-transfection	Day 20 post-infection
SIVmac239	ACATGTCTCATTTTATAAAAGAAAAGGGGGGACTGGAAGGGATT			
SIV/Ubox-sub4	..........GACGTC.....			
SIV/Ubox-sub4 (A)	..........GACGTC.....	5/9	2/9	none
SIV/Ubox-sub4 (B)	..........GACGT......	2/9	6/9	9/10
SIV/Ubox-sub4 (C)	..........GA.GTC.....	1/9	none	none
SIV/Ubox-sub4 (D)	..........GAAGTC.....	1/9	1/9	1/10

Four types of U-box sequences (A–D) were recovered from CEMx174 cell cutures transfected or infected with the mutant SIV/Ubox-sub4. Virus stock for infection was prepared from cell culture supernatant 35 days post-transfection. Sequence of the polypurine tract is underlined.

Table VII. Sequence rearrangements in the proviral DNA recovered from cell cultures transfected with the mutant SIVΔUbox

Virus	Sequence	Origin
	9408 9444	
SIVmac239	TGAGTTACAAATTGGCAATAGACATGTCTCATTTTAT ppt	wild-type
SIVΔUbox	.....C------ ppt	constructed mutant
SIVΔUbox-tr1 (9/9)	.....C------ ppt	recovered 21 days after transfection
SIVΔUbox-tr2 (1/9)	.....C------ ppt	recovered 18 days after transfection
SIVΔUbox-tr2 (2/9)	.....T------ ppt	
SIVΔUbox-tr2 (5/9)	.....---------- ppt	
SIVΔUbox-tr2 (1/9)	.....----------.C------ ppt	
SIVΔUbox-tr3 (3/9)	.....C------ ppt	recovered 17 days after transfection
SIVΔUbox-tr3 (1/9)	.....T------ ppt	
SIVΔUbox-tr3 (4/9)	.....T..C------ ppt	
SIVΔUbox-tr3 (1/9)	.....----------.C------ ppt	

Results from three independent transfections are presented. Number of clones sequenced is shown in parentheses. Dashes stand for deletions.

tRNA binds to a specific segment adjacent to the 3′-end of U5, called the primer-binding site (PBS). Initiation of plus-strand synthesis takes place in one major and several minor sites that utilize short segments of the RNA genome (Finston and Champoux, 1984; Omer *et al*., 1984; Resnick *et al*., 1984; Smith *et al*., 1984a,b; Huber and Richardson, 1990; Luo *et al*., 1990; Pullen and Champoux, 1990; Charneau *et al*., 1992; DeStefano *et al*., 1993, 1994;

Table VIII. Sequence rearrangements in the polypurine tract after infection of CEMx174 cell cultures with the mutant SIVΔUbox-tr1

Virus	Sequence	Origin	Length of novel poly(A)
	9427 9463		
SIVmac239	AGACATGTCTCA----------TTTTATAAAAGAAAAGGGGGGAC∧TG	wild-type	
SIVΔUbox	..........C----------AAAAGAAAAGGGGGGAC∧TG	constructed mutant	
SIVΔUbox-tr1 (9/9)	..........C----------AAAAGAAAAGGGGGGAC∧TG	recovered 21 days after transfection	
SIVΔUbox-inf1 (1/9)	....G.....C----------AAAAGAAAAGGGGGGAC∧TG	recovered 20 days	
SIVΔUbox-inf1 (1/9)	..........C----------AAAAAAAAAAAAAAAAAAGGGGGGAC∧TG	after infection	17
SIVΔUbox-inf1 (4/9)	..........C----------AAAAAAAAAAAAAAAAAAGGGGGGAC∧TG		20
SIVΔUbox-inf1 (1/9)	..........C-AAAAAAAAAAAAAAAAAAAAAAAAAAGGGGGGAC∧TG		27
SIVΔUbox-inf1 (2/9)	..........CAAAAAAAAAAAAAAAAAAAAAAAAAAGGGGGGAC∧TG		28
SIVΔUbox-inf2 (1/9)	..........C----------AAAAGAAAAGGGGGGAC∧TG	recovered 17 days	
SIVΔUbox-inf2 (3/9)	..........C----------AAAAAAAAAAAAAAAAAAGGGGGGAC∧TG	after infection	17
SIVΔUbox-inf2 (3/9)	..........C----------AAAAAAAAAAAAAAAAAAGGGGGGAC∧TG		18
SIVΔUbox-inf2 (1/9)	..........C---------AAAAAAAAAAAAAAAAAAAGGGGGGAC∧TG		19
SIVΔUbox-inf2 (1/9)	..........C--------AAAAAAAAAAAAAAAAAAAGGGGGGAC∧TG		20

Results from two separate infections are shown. The viral stock for the infection was prepared 18 days after transfection. The clones from the first infection were derived by two independent PCRs. Number of clones for each sequence related to total number of clones derived from the corresponding culture is shown in parentheses.∧ – start of LTR. Dots stand for identities in sequence, dashes stand for deletions.

Pullen *et al*., 1993; Fuentes *et al*., 1995; Heyman *et al*., 1995; Lauermann *et al*., 1995; Powell and Levin, 1996; for reviews see Coffin, 1990; Champoux, 1993; Telesnitsky and Goff, 1993). The major primer for plus-strand synthesis is located immediately upstream of the U3 region and is called the polypurine tract (PPT). Recent reports point to an even greater complexity of reverse transcription, supporting the participation of additional PPT-like sequences in the synthesis of plus-strand and suggesting this process is discontinuous (Charneau *et al*., 1992; Heyman *et al*., 1995; Wilhelm *et al*., 1997). It also appears that in addition to the enzymes associated with reverse transcriptase, other proteins, namely HIV-encoded NC7 and tat, play a significant role in reverse transcription (Wu *et al*., 1996; Guo *et al*., 1997; Harrich *et al*., 1997). Evidence has also been presented to support the theory that mutations in close proximity to the PBS (in the U5 and leader regions) may also influence viral replication (Li *et al*., 1997; Liang *et al*., 1997).

A conserved uridine-rich sequence located immediately upstream of the PPT in various HIV-1 strains has been noted previously (Powell and Levin, 1996). Interestingly, when the plus-strand primer itself was sequenced *in vitro* from the reverse transcription system of HIV, it contained the upstream uridines (usually four, but in some cases two) in addition to the PPT (Huber and Richardson, 1990). Sequence analysis indicates that the U-box-like element upstream of the PPT is present not only in HIV and SIV (Table I), but in the vast majority of retroviruses and retroviral elements (Table II). Conservation such as this suggests a common functional role throughout the retroviral family. Enzymatic assays have been used to investigate a possible role for these sequences in reverse transcription of HIV-1 but these experiments failed to reveal any contribution of this element to the second strand initiation process (Powell and Levin, 1996).

In this paper we present data documenting a profound

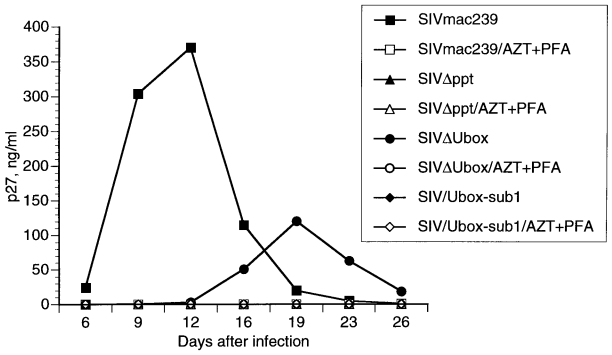


Fig. 3. Replication of COS-1-derived SIVmac239 U-box and mutant PPT viruses in CEMx174 cells. Virus stocks of SIVmac239, SIVΔppt, SIVΔUbox and SIV/Ubox-sub1 were prepared by transient transfection of COS-1 cells, assayed for p27 and used for infection of CEMx174 cells in the presence (closed symbols) or absence (open symbols) of inhibitors of reverse transcription (AZT and PFA) as described in Materials and methods.

effect of U-box mutations on SIVmac239 replication *in vitro*. Consistent results were obtained when different mutant viral strains were used. It appears that the effects of the U-box are determined principally by the three bases immediately upstream of the PPT and that the presence of thymidine in at least two of these three positions is significant. Point mutations within the U-box affected viral replication more profoundly than deletions. We tested three distinct mutants bearing point changes to three or more bases immediately upstream of the PPT (Ubox-sub1, Ubox-sub2 and Ubox-sub4). A total of 12 transfections with these three mutants resulted in virus growth in only two separate experiments and only after a profound delay. In both cases, changes occurred in the mutated sequence resulting in the emergence of two thymidines immediately upstream of the PPT. The ΔUbox mutant, in which six nucleotides immediately upstream of PPT were deleted,

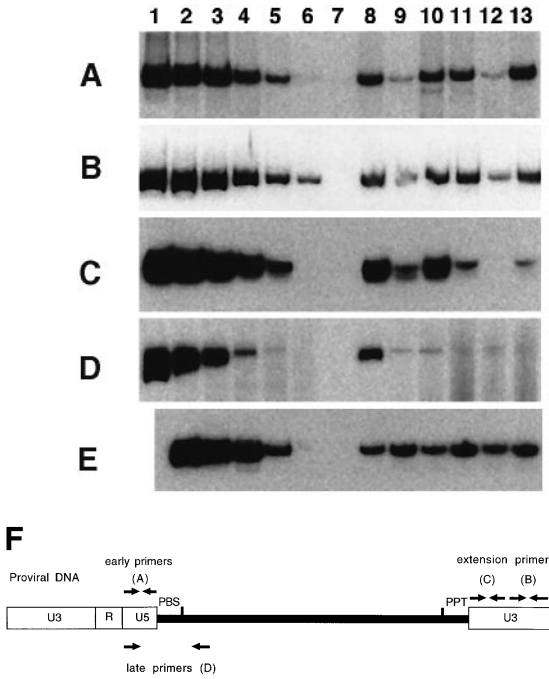
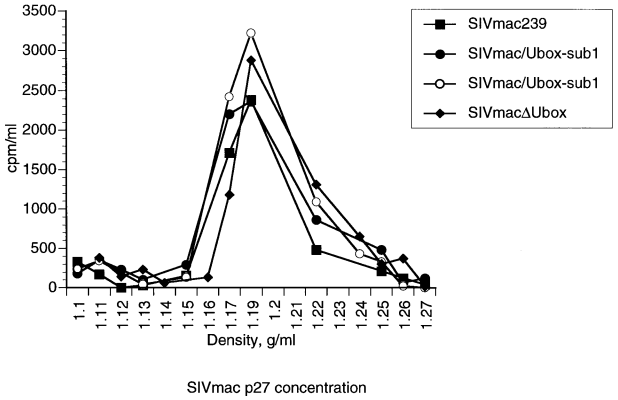


Fig. 4. SIVmac239 U-box mutants are defective in reverse transcription. CEMx174 cells were infected with virus derived from transient transfection. Viral sequences were PCR-amplified with primers specific for early (**A**), extended (**B** and **C**) or late (**D**) reverse transcripts 20 h post-infection. Results of amplification of β-globin sequences are presented in (**E**). Lanes 1–7 contain 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, 10 and 0 copies of proviral DNA, respectively (A–D); in E lanes 2–7 contain 8, 3.2, 1.6, 0.4, 0.1 and 0 μg of cellular DNA correspondingly. Lanes 8–13 contain DNA from cultures infected with SIVmac239, SIVmac239 (RT-inhibitors treated), SIVΔppt, SIVΔUbox, SIVΔUbox (RT-inhibitors treated) and SIVΔUbox-sub1, respectively. (**F**) The position of primer pairs used for the amplification of early, extended and late reverse transcripts. The early primer pair will amplify the newly-synthesized viral DNA immediately after initiation and before first-strand transfer. Extension primer pairs will amplify newly-synthesized viral DNA immediately after first strand transfer (B) and after its further elongation into the U3 region (C). Late primers will amplify newly-synthesized viral DNA after its elongation past the PBS/leader sequence. Positions of primers are shown by arrows.



SIVmac p27 concentration

wild-type	-	-	-	0.1	0.3	0.05	-	-
Ubox-sub1	-	-	-	0.1	0.4	0.18	-	-
Ubox-sub1	-	-	-	0.2	0.7	0.3	-	-
ΔUbox	-	-	-	0.2	0.4	0.05	-	-

Fig. 5. Incorporation levels of [^{3}H]uridine into wild-type SIVmac239 and U-box mutants corresponds to concentration of capsid viral protein, p27. Levels of ^{3}H-radioactivity in sucrose gradient fractions are presented. Concentration of p27 in corresponding gradient fractions is shown below the radioactivity curves. The results of two independent simultaneous transfection/gradient centrifugations are shown for SIV/Ubox-sub1.

replicated *in vitro* but with a considerable delay. However, when two additional thymidines were mutated in the strain ΔUbox (ΔUbox-sub1), replication was again strongly suppressed.

What stage of SIV replication is affected by U-box mutation? We have not detected any change in viral RNA synthesis and packaging with these mutants; our data indicate that the block to viral replication in SIV U-box mutants occurs in the early stages, specifically during reverse transcription. This block occurs between the first-strand jump and the start of second-strand synthesis. While Powell and Lewin (1996) did not detect an effect of U-box sequences on the priming and extension of HIV second-strand (plus-strand) DNA in biochemical assays, their results do not necessarily conflict with ours, as biochemical assay conditions do not always represent what is occurring *in vivo*. The exact role of U-box sequences in facilitating synthesis through the end of U3 and the PPT remains to be elucidated. One intriguing possibility, consistent with the compensatory PPT expansion we observed, is that U-box sequences may help to prevent misalignment and deletions through the extensive runs of purines in this region.

Materials and methods

Construction of mutated viruses

Mutants of SIVmac239 nef-open which bear changes in the PPT and U-box sequences were created by site-specific mutagenesis. Wild-type SIVmac239 was previously cloned in two parts and is carried on plasmids p239SpSp5′ (5′-clone) and p239SpE3′ (3′-clone), which contain left and right halves of the genome respectively (Kestler *et al*., 1990; Regier and Desrosiers,1990). In this work we have used a modified 3′-clone of SIVmac239 (kindly provided by Z.Du, Harvard Medical School), in which an additional *Eco*RI site was introduced just after the viral sequence, and from which the cellular DNA sequences were eliminated. The resulting 3834 bp *Sph*I–*Eco*RI viral DNA fragment was cloned into plasmid pSp72. The use of this construct significantly simplified subcloning and recloning procedures. The viral sequence in this 3′-clone is identical to that of the 3′-clone used previously (Kestler *et al*., 1990; Regier and Desrosiers, 1990).

The 1052 bp *Sac*I–*Eco*RI fragment of the 3′-clone was then subcloned into pSp72 and used to introduce site-specific mutations. PCR-directed mutagenesis was employed (Higuchi *et al*., 1988) using one or two mutagenic primers and two terminal primers in each reaction. Terminal primers spanned two *Bsu*36I sites, which are unique to the reconstructed 3′-subclone. The resulting 507 bp fragment was digested with this enzyme and inserted into the *Sac*I–*Eco*RI subclone. In some instances, the mutation-containing PCR product was initially cloned into a blunt-end cloning vector (PCRII, Invitrogen Corp., San Diego, CA) or pUC18/SmaI (Pharmacia, Piscataway, NJ), and upon sequence verification, recloned back into the *Sac*I–*Eco*RI subclone. The *Sph*I–4*Sac*I fragment of proviral DNA was then inserted into this mutated subclone, creating a full 3′-clone of SIVmac239. The mutated clones were multiply sequenced through the amplified region and all the restriction sites used in mutagenesis. To achieve efficient transfection of COS-1 cells, the pBRmac239 plasmid was used, which bears the full-size SIVmac239 genome (kindly provided by T.Kodama, Oregon Primate Research Center). *Sph*I–*Eco*RI fragments of mutant 3′-clones ΔUbox, Ubox-sub1 and Δppt were directly inserted into the plasmid to create full-size mutated SIV clones.

Molecular cloning

All routine cloning procedures were essentially performed as described earlier (Ilyinskii *et al*., 1994; Ilyinskii and Desrosiers, 1996). Epicurian Coli XL1-Blue MRF Supercompetent Cells (Stratagene Cloning Systems,

La Jolla, CA) were used for transformation and plasmid growth. Each of the resulting plasmids was sequenced at least twice through the viral insert. PCR-amplified viral fragments from infected culture cell lines were treated with polynucleotide kinase and cloned into a pUC18/SmaI-cut vector (Pharmacia) or PCRScript (Stratagene) according to manufacturers' recommendations.

Primers

All nucleotide primers were custom-made by Gibco-BRL (Grand Island, NY). Terminal primers used in PCR mutagenesis were 5′-CGAGAAGTCCTCAGGACTGA-3′ (9040–9059) and 5′-TTCCTGGTCCTGAGGTGTAA-3′ (9569–9550). Mutagenic primers used were: 5′-GGCAATAGACATGTCTCCAAAAGAAAAGGGGGGA-3′ and 5′-TCTTTTGGAGACATGTCTATTGCCAATTTGTAAC-3′ (for the creation of the ΔUbox clone), 5′-TGGCAATAGACATGTCTCCCCCCGCAAAAGAAAA-3′ and 5′-TTTTGCGGGGGGAGACATGTCTATTGCCAATTTGTAACT-3′ (Ubox-sub1), 5′-TTGGCAATAGACATGTCTCATCCCGCAAAAGAAAAGGGGGGACT-3′ (Ubox-sub2), 5′-TTGGCAATAGACATGTCTCCCCCTATAAAAGAAAAGGGGGGAC-3′ (Ubox-sub3), 5′-TTGGCAATAGACATGTCTCAGACGTCAAAAGAAAAGGGGGGACT-3′ (Ubox-sub4), 5′-CAAATTGGCAATAGACATGGCCCCAAAAGAAAAGGGGGG-3′ (ΔUbox-sub1), 5′-ATAGACATGTCTCATTTTATACTGGAAGGGATTTATTACA-3′ and 5′-CCCTTCCAGTATAAAATGAGACATGTCTATTGCCAATTTGTAACTC-3′ (Δppt). Primers 5′-AGATGATGACTTGGTAGG-3′ (9355–9372) and 5′-GGGACTAATTTCCATAGCCA-3′ (9612–9593) were used for sequencing through the amplified region. Primers 9040–9059 and 9569–9550 were also used for amplification of the PPT/U-box region from infected cells. Primer pairs 5′-GCTAGTGTGTGTTCCCATCTCT-3′ (696–717), 5′-CAGAAAGGGTCCTAACAGACCA-3′ (785–764) (both located in the U5 region), 5′-CTTAACATGGCTGACAAGAAGG-3′ (375–396), 5′-ATTTATACATCAAGAAAGTGGG-3′ (494–473) (3′ part of the U3 region), 5′-GGGATTTATTACAGTGCAAGAAGA-3′ (9467–9490) 9569–9550 (5′ part of the U3 region) and 696–717, 5′-ATAGGAGCACTCCGTCGTGGTTGG-3′ (923–900) (overlapping U5 region and leader sequences) were used for the quantitative PCR amplification of SIVmac early, extended and late reverse transcripts in infected cells. β-globin primers used in these experiments were identical to those in Mori *et al*. (1993).

PCR amplification and DNA sequencing

PCR amplification and DNA sequencing were performed essentially as described earlier (Ilyinskii *et al*., 1994; Ilyinskii and Desrosiers, 1996). A thermal cycler from MJ Research (Watertown, MA) was used for PCR amplification. In quantitative PCR amplification, the forward primer was ^{32}P-labeled in a kinase reaction using [γ-^{32}P]ATP. DNA sequencing was performed using an ABI Prizm automated sequencer. Sequencing reactions were performed with the help of AmpliTaq FS sequencing kit (Perkin Elmer-Cetus, Norwalk, CT), used according to manufacturer's recommendations on either MJ Research or Hybaid Omnigene (Sun-Bioscience Inc., Branford, CT) thermal cyclers.

Nucleotide sequence analysis

Nucleotide sequences were analyzed and aligned using MacVector version 4.1.4 software (International Biotechnologies Inc., New Haven, CT). The SIVmac239 sequence has been published previously (Regier and Desrosiers, 1990). Retrovirus and retroelement sequences were taken from DDBJ/EMBL/GenBank (Bilofsky and Burks, 1988) [accession numbers: M19921 (HIV-1, NL4-3), K02013 (HIV-1, LAI), K03455 (HIV-1, HXB2), K02007 (HIV-1, SF2), M30502 (HIV-2, BEN), J03654 (HIV-2, NIHZ), M15390 (HIV-2, ROD), M27470 (SIVmnd), X07805 (SIVagm), M37980 (AMV), K02120 (BLV), M32690 (BIV), M33677 (CAEV), M87581 (EIAV), K01208 (FeLV), M25381 (FIV), Z11128 (Fr-MuLV), M14123 (HERV-K10), M12349 (MPMV), J02262 (Mo-MuLV), M15122 (MMTV), X16839 (MuEV), J02342 (RSV) and Z48149 for chromosome 15 of *Streptomycin cerevisiae* which includes the yeast retrotransposon sequence, Ty1].

Cells

Rhesus monkey PBMC, CEMx174 and COS-1 cells were maintained as described earlier in RPMI 1640 or Dulbecco's modified Eagle's medium (Gibco-BRL Laboratories, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum and penicillin–streptomycin (and additional 10% IL-2 for PBMC) (Ilyinskii *et al*., 1994; Ilyinskii and Desrosiers, 1996). The culture medium was changed twice weekly.

Preparation of virus stocks

Pelleted CEMx174 cells were transfected with *Sph*I-digested 5′- and 3′-SIVmac plasmids by a DEAE-dextran procedure (Naidu *et al*., 1988). For stock preparation, virus was harvested at or near the peak of virus production, filtered and stored in aliquots at –80°C. Stocks were assayed for the concentration of SIV p27 gag antigen with a commercially available antigen-capture kit (Coulter Corp., Hialeah, FL). COS-1 cells were transfected with full-size SIV-genome bearing plasmids with the help of a LipoTAXI™ Mammalian Transfection Kit (Stratagene) used according to manufacturer's recommendations. Virus-containing cell culture medium was collected 2–4 days after transfection.

Virus replication in vitro

CEMx174 cells, split 1:3 the previous day, were either transfected as described above or infected with an aliquot of SIVmac containing 2 ng of viral p27 protein. Activated PBMCs were infected with the same amount of SIVmac upon stimulation with phytohemagglutinin (Sigma, St Louis, MO) as described earlier (Ilyinskii *et al*., 1994; Ilyinskii and Desrosiers, 1996). Cell supernatants were sampled, clarified, and assayed for SIV p27 by antigen capture.

Isolation of cellular DNA

Infected CEMx174 cells were pelleted, washed twice with serum-free RPMI and PBS and lysed in a small volume (50–100 μl). DNA was isolated in a single microcentrifuge tube for each sample with the help of a HRI AmpPrep™ kit (HRI Research Inc., Concord, CA) and subjected to PCR amplification as described above.

Detection of viral DNA

To avoid contamination with SIV DNA, COS-derived viral stocks were pretreated with DNase I (10 μg/ml, 1 h, 20°C). Equal amounts of CEMx174 cells were then incubated with COS-derived stocks of wild-type and mutant SIVmac239 (1 ng each of p27) for 2 h, washed twice in serum-free RPMI medium and incubated in standard conditions for 16–20 h. Control CEMx174 cell samples were treated with reverse transcription inhibitors AZT (50 μM) and PFA (100 μM) 8–10 h prior to infection (Mori *et al*., 1993). Infected cells were washed in RPMI and PBS and the total DNA was prepared as described above. These samples were used for the detection of viral reverse transcripts with the help of quantitative PCR. The integrity and quantity of DNA samples were confirmed by control amplifications with β-globin primers. Primer pairs to amplify sequences located inside U5, U3 or U5/leader regions were used to detect early, extended and late products of reverse transcription as described above. Sets of standard reactions were consistently run in parallel with experimental amplification.

[^{3}H]uridine labeling and gradient centrifugation of retroviral particles

COS-1 cells to be transfected with full-genome SIV plasmids were pre-incubated with 100 μCi of [^{3}H]uridine for 16 h. Transfected cells were kept on [^{3}H]uridine-containing culture medium. Control cultures were treated with mRNA synthesis inhibitor, α-amanitin (Van Lint *et al*., 1996). Culture medium samples were collected 48 h after transfection, cleared (40 min at 10 000 r.p.m.) and the labeled virus pelleted by ultracentrifugation [3 h at 25 000 r.p.m. in a SW27 rotor (Beckman)]. Crude virus pellets were loaded onto 20–60% continuous sucrose gradient and centrifuged for 2.5 h in a SW41 rotor (37 000 r.p.m.). The gradient was then split into 250 μl fractions, of which 10 μl were then used for the measurement of the radioactive emission. Ecolume™ scintillation fluid (ICN Pharmaceuticals, Irvine, CA) was added to each aliquot which was subsequently counted in a Delta 300 β-counter (Searle). The amount of p27 in each sucrose fraction was determined by antigen capture assay as described above. The sensitivity of p27 detection in 40% sucrose solution was shown not to differ from standard conditions (data not shown).

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