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Rates of Shutdown of HIV-1 into Latency: Roles of the LTR and *tat/rev/vpu* Gene Region

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CEM T-cells chronically infected with most HIV-1 isolates gradually cease virus production over a 4–6 week period. This is due to slow shutdown of virus replication in the majority of the cells, leading to latent infections. We identified one HIV-1 isolate (HIV_{213}) which shut down into latency at a rate much slower than most HIV strains, requiring more than 12 weeks for the majority of the cells to become nonproductive. This indicated that genes of the virus influence the rate of shutting down, or alternatively, the length of time chronically infected cells produce virus. The viral gene(s) influencing differential rates of shutdown were mapped using chimeric viruses composed of the HIV_{213} genome substituted with various restriction fragments from HIV_{MCK}, which rapidly progresses into latency. We found that the 3′ region of the LTR was the major determinant influencing the rate of shutdown, but the *tat/rev/vpu* region also slightly influenced this phenotype. These data show that at least these two genomic regions can influence the duration of virus production in chronically infected cells and that polymorphisms in these regions result in phenotypically divergent viruses which go into latency at different rates. It is also possible that this viral property may be an important determinant of clinical outcomes.

INTRODUCTION

Human immunodeficiency virus (HIV-1) belongs to the Lentivirus family of retroviruses, the prototypes of which are characterized by their ability to cause slowly progressive disease with prolonged periods of subclinical infection (Narayan and Clements, 1988; Narayan *et al.,* 1988). Unlike most simple oncoviruses, Lentiviruses such as Visnavirus and Equine Infectious Anemia Virus (EIAV) routinely establish latent infections, and this has been implicated in their slow pathogenesis (Cheevers and McGuire, 1985; Haase *et al.,* 1978; Narayan and Clements, 1988). The ability of a virus to go into latency is considered an important mechanism for escaping the antiviral immune responses, thereby allowing persistence. Many viruses which establish persistent infections are able to go into latency (Mahy, 1985). Recent *in situ* PCR and hybridization analyses of HIV proviral DNA and RNA in patient tissues have confirmed that HIV also establishes a significant extent of nonproductive infections. Up to 20% of the lymphocytes in lymph nodes of asymptomatic infected subjects harbor HIV proviral DNA, while very few cells (0.1–0.001% or less) appear to be replicating virus (Embertson *et al.,* 1993; Harper *et al.,* 1986). Plasma viral levels usually remain low during the asymptomatic period, but increase as the disease develops (Clark *et al.,* 1991; Gupta *et al.,* 1993; Ho *et al.,* 1989; Michael *et al.,* 1995; Schnittman *et al.,* 1990). These observations led to speculation that HIV can be reactivated from latency like herpesvirus (see Garcia-Blanco and Cullen, 1991, for review). However, little evidence exists for significant reactivation of latent HIV *in vivo.* Nevertheless, HIV's ability to go into latency could be an important mechanism by which it persists in face of the host's antiviral immune response.

In vitro, the majority of chronically-infected HIV-producing $CD4^+$ T- or monocyte-macrophage cell lines gradually cease production over time. One early study showed in a cell line which contained a subpopulation of $CD4^-$ (uninfectable) cells, that the latter overgrew the chronically infected culture, eventually resulting in a population of uninfected cells (Folks *et al.,* 1986). Consequently, most studies of HIV latency have used certain cell clones that are already latently infected (Butera *et al.,* 1994; Clouse *et al.,* 1989; Folks *et al.,* 1988; Folks *et al.,* 1986; Pomerantz *et al.,* 1990; Stanley *et al.,* 1989). However, in cell lines which do not contain uninfectable cells, cessation of HIV production during chronic infection is predominantly due to gradual shutdown of virus production in the majority of infected cells (Li *et al.,* 1996). Use of populations of acutely infected cells rather than latently infected cell clones to study what happens during chronic infection will make it easier to study the events as the virus shuts down expression and enhances the reliability of such studies by examining events which occur in the majority of the cells.

One such recent study by Mustafa and Robinson impli-

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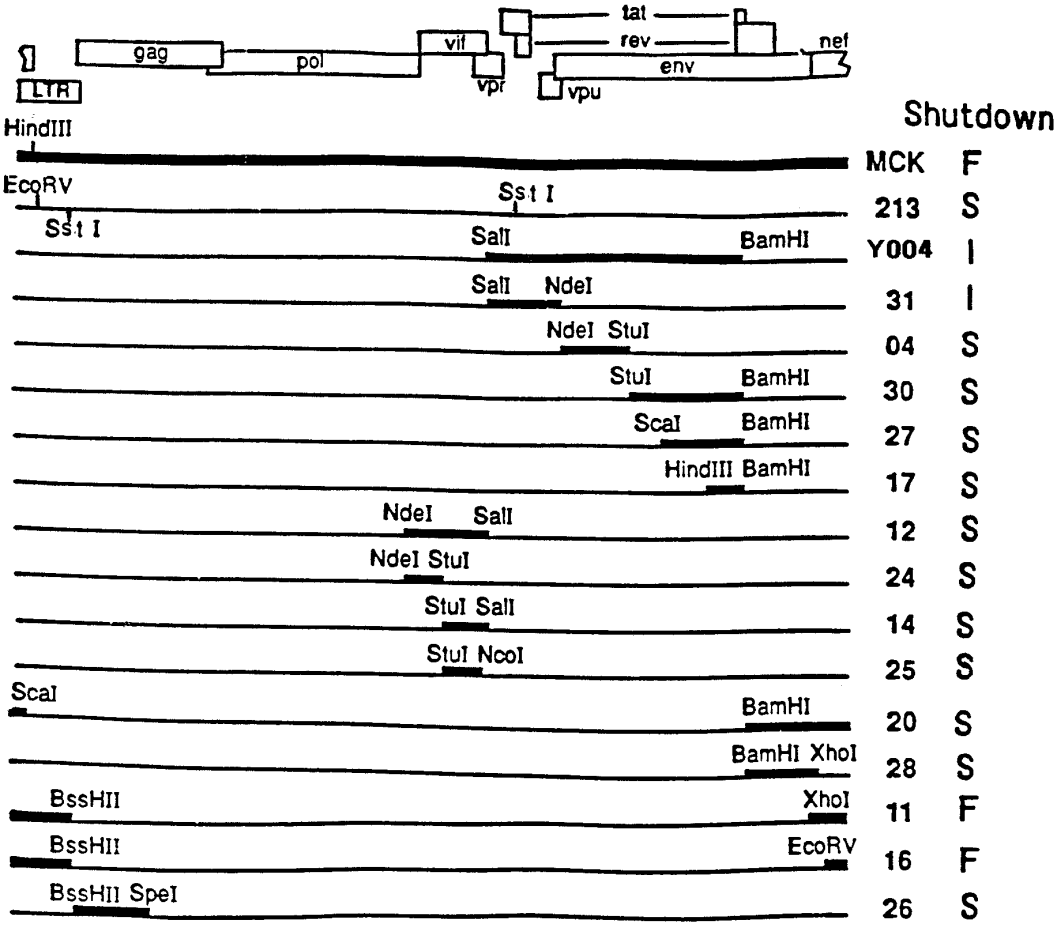

FIG. 1. Genomic constructs of chimeric viruses. Different restriction fragments of the cloned HIV_{213} genome (thin line) were replaced with the same restriction fragments from cloned HIV_{MCK} (thick line). Restriction sites that differ between the two viruses are shown. These hybrid genomes were then transfected into CEM cells and propagated to make virus stocks. The summarized results of the study are also shown: F, fast shutdown; S, slow shutdown; I, intermediate shutdown rate.

cated various accessory genes of HIV in influencing the length of time virus expressed in transfected H9 cells (Mustafa and Robinson, 1993). They found that there was an inverse correlation between the degree of cell injury and the length of time virus expressed, which led to the suggestion that various accessory genes interplayed leading to complex interactions impacting on cell injury and cell signaling for growth. Importantly, this study examined the role of cell injury, for dead or dying cells will not produce new virus. This study was thorough, but it's results were quite complex because of the complexity of the viruses used (HXB-2 having several nonfunctioning accessory genes and NL4-3 composed of sequences from two very divergent HIV strains), the confounding cytopathicity, and because transfection rather than infection was employed. Another study by Mikovits and coworkers (Mikovitz *et al.,* 1990) examined chronic infections of THP-1 promonocytic cells and found that, depending on the virus strain used and multiplicity of infection (m.o.i.), the cell population could become either latently infected, have restricted low level expression, or be continuous producers. It is not clear, however, how the various factors (viral genes, host factors, m.o.i., etc.) each influence chronic viral expression in the majority of infected cells.

We also studied what happens to HIV expression during chronic infection of T-cell populations and found that the majority of cells in PHA-stimulated $CD4^+$ PBLs and the CEM T-cell line chronically infected with most HIV isolates gradually cease producing virus by 3–6 weeks postinfection (Li *et al.,* 1996). Infected H9 cells take even longer, but also slowly shut down the virus (unpublished data). We further found that one HIV-1 isolate (HIV_{pm213} or HIV_{213}) we previously isolated and characterized (Cloyd and Moore, 1990) required more than 12 weeks to cease production of virus in chronically infected CEM cells (Li *et al.,* 1996). This demonstrated that, similar to Mustafa and Robinson's study, sequences of HIVs could influence the length of time of chronic virus production or alternatively the rate that chronically infected cells shut down HIV production. This, in turn, allowed us to attempt to identify the gene(s) of HIV which influence

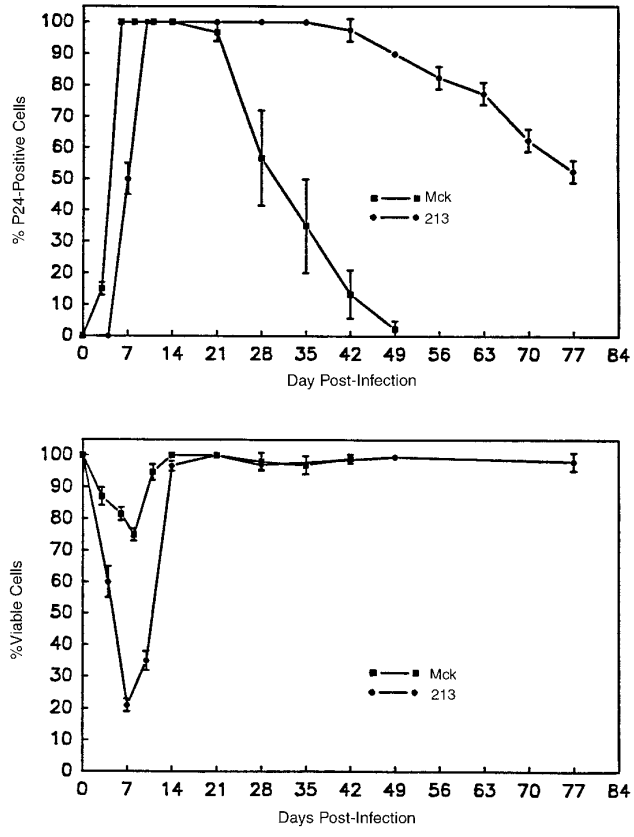

FIG. 2. Kinetics of HIV expression in chronically infected CEM cells. CEM cells were chronically infected with HIV_{213} (filled circles) or HIV_{MCK} (filled squares). The percentage of cells producing virus was determined by fixed-cell immunofluorescence for p24 antigen for a period of up to 3 months. During the experimental period, the viability of the chronically infected cells was determined by trypan-blue exclusion (HIV_{213}, open circles; HIV_{MCK}, open squares).

the rate of shutdown. We cloned full-length infectious genomes of this virus (HIV_{213}), and HIV_{MCK}, which shuts down rapidly but is lowly cytopathic (Cloyd and Moore, 1990), and constructed chimeric viruses by substituting common restriction fragments from HIV_{MCK} into HIV_{213}. Stocks of infectious chimeric viruses were made, and the time required for CEM cells chronically infected with these viruses to shut down virus production was determined and compared with that of the parental viruses. We found that the 3′ sequences of the LTR, but not *nef,* profoundly influenced the rate of virus shutdown. Independently, the *tat/rev/vpu* region also influenced this property, but weakly. Thus, it appears that at least these two HIV genomic regions can be involved in determining the rate of shutdown of HIV.

MATERIALS AND METHODS

Viruses and cells

Isolation and partial characterization of HIV_{213} (HIV_{pm213}) and HIV_{MCK} have been reported previously (Cloyd and Moore, 1990). The cell lines used in this study were CEM and H9, human CD4-positive T-cell lines. The cells were grown as suspension culture in RPMI-1640 medium plus 5% fetal bovine serum and 5% Fe^{2+}-transferrin supplemented calf serum, and penicillin and streptomycin. The cells were incubated in humidified 5% CO_2 at 37°.

Cloning of HIV_{pm213} and HIV_{MCK} proviruses

λ genomic libraries of the HIVs were constructed from unintegrated circular forms of HIV genomic DNA. Low molecular weight DNA was obtained from acutely infected 2×10^8 H9 cells (m.o.i. = 1) by the Hirt method (Hirt, 1967). Both genomes of HIV_{pm213} and HIV_{MCK} were confirmed to have a unique *Bam*HI site by restriction analysis and Southern blot hybridization with ^{32}P-labeled pBH10 HIV probe. The Hirt low molecular weight DNA, cut with *Bam*HI, was ligated to *Bam*HI arms of EMBL3 λ vector and packaged with a commercial packaging kit. 5×10^5 phages were screened by *in situ* plaque hybridization (Hirt, 1967). After plaque purification of positive plaques, single plaques were amplified. The cloned HIV inserts were then transferred to the pUC18 plasmid vector for restriction mapping. Thirty-five restriction sites were localized by 25 different restriction enzymes and five noncutting enzymes were also identified. Except for a few sites (Fig. 1), most of the restriction sites were common between HIV_{MCK} and HIV_{pm213}, even though many of the biological properties of these viruses were different (Cloyd and Moore, 1990). The genomic size of both was 9.1 kb and each contained one LTR.

Construction of chimeric genomes between HIV_{pm213} and HIV_{MCK}

Based on the restriction maps, common restriction fragments of HIV_{213} and HIV_{MCK} DNAs were isolated from the plasmid by digesting the DNA with restriction enzymes and separating them by gel electrophoresis, followed by electro-elution. A specific small portion of the HIV_{213} genome was substituted with the corresponding HIV_{MCK} fragment and ligated. Fifteen different chimeric genomes were eventually constructed, each possessed the HIV_{213} backbone containing an HIV_{MCK} DNA fragment substituted for its own in various regions of the genome (Fig. 1). We initially focused on the 3′ half of the genome and then made chimeras in the LTR and 5′ *gag* regions. Since the *gag* and *pol* of these two viruses were very similar from sequence analyses, we did not make chimeras encompassing 3′ half of *gag* or *pol.*

Transfection and generation of virus stocks

Since the circular form of HIV DNA was linearized and ligated to the vector for cloning, the genomic structure was interrupted by the vector. To reconstruct the genome

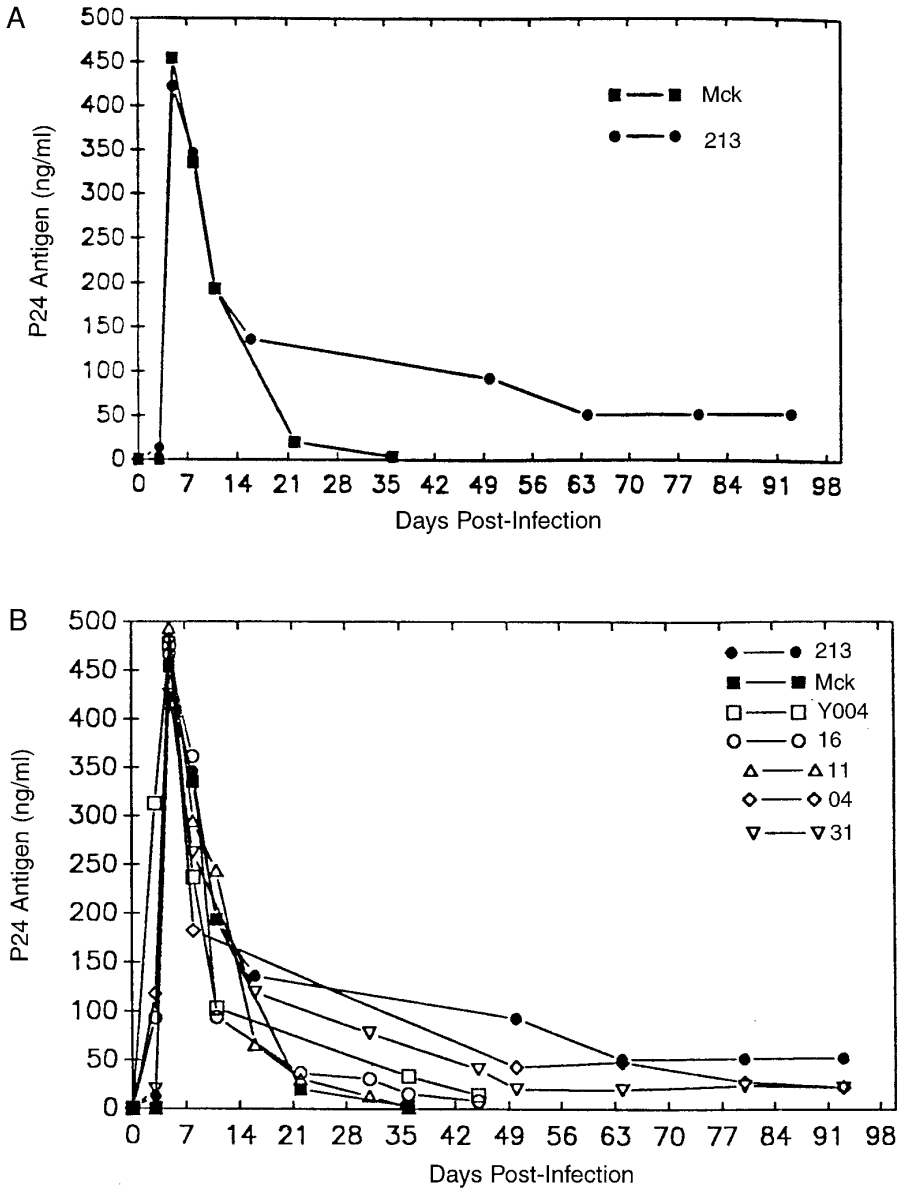

FIG. 3. Parental and chimeric virus production. P24 antigen released into the medium was quantitated by antigen-capture EIA (Coulter). CEM cells were infected with HIV_{213} (filled circles) of HIV_{MCK} (filled squares) (A) or with chimeric viruses (B) for 24 hr and then rinsed thoroughly before further culturing. The cells were subcultured routinely and aliquots of the culture fluid were harvested weekly and stored at −70° until analyzed by antigen-capture EIA.

in its correct form, the genome had to be removed from the plasmid and recircularized. The plasmid DNA containing the HIV genome was cut with *Bam*HI, *Xba*I, and *Sma*I. There are no *Xba*I and *Sma*I restriction sites in either HIV_{213} and HIV_{MCK}. The *Xba*I site is localized at the third basepair from the left side of the *Bam*HI site and the *Sma*I is at 6 bp distance from the right side of the *Bam*HI site in the multiple cloning site of pUC18. The small oligonucleotides produced from the digestion with those three restriction enzymes were removed by precipitating the digested DNA in TE buffer containing 2.5 *M* ammonium acetate and 2 vol of 100% ice-cold ethanol. Therefore, only the HIV inserts would possess compatible ends for ligation. The DNA at a concentration of 5 μg/ml was ligated for recircularization. Three microgram of circularized DNA was then transfected into 4×10^6 CEM cells by electroporation at 200 V, 500 μF in a gene pulsar (Bio-Rad). Production of progeny virus was monitored by *in situ* immunostaining of p24 viral antigen in the transfected cells as described elsewhere (Cloyd and Moore, 1990). The cloned parental genomes and all the hybrid genomes produced viruses, and stocks were made from the transfected CEM cells when the cells were 90–100% p24 positive.

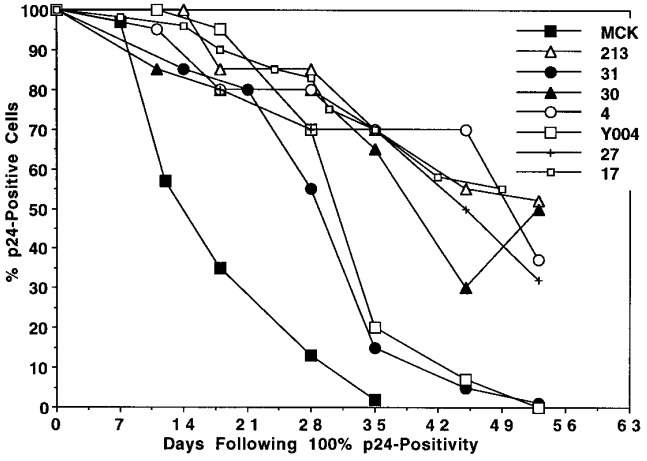

FIG. 4. Kinetics of expression of chimeric viruses possessing substitutions in 3′ region of genome. Molecularly cloned HIV_{213} and HIV_{MCK} were used. The percentage of p24-positive cells was determined as described in the legend of Fig. 2.

Virus infection and virus assays

Virus stocks were pretitered by serial half-log dilutions on C8166 cells as described previously (Cloyd and Moore, 1990). To study chronic virus production, 1×10^5 CEM cells per well in 24-well plates were infected with each virus at m.o.i. of 0.5–1.0. Following the peak of acute CPE at 5–8 days postinfection, the surviving cells became chronic producers. The percentage of cells producing virus was determined by fixed-cell immunofluorescence for p24 at weekly time intervals postinfection, and the progeny virus produced was quantitated by determining the amount of p24 HIV core antigen (by a Coulter antigen-capture EIA) in culture supernatants. The HIV-producing cells were maintained in optimum culture conditions throughout the experiment, because poor conditions accelerate the rate that chronically infected cells shutdown.

DNA sequencing

The DNA was sequenced by the chain-termination DNA sequencing method (Sanger *et al.,* 1977). Plasmid DNA was prepared by rapid plasmid preparation methods with extra phenol/chloroform extraction. [^{35}S]dCTP was used for sequencing reaction using a commercially available sequencing kit (Sequenase version 2, United States Chemicals). Electrophoresis was done at constant 60 watts and 1800 V. Gel processing and autoradiography was performed as described elsewhere (Sambrook *et al.,* 1989). The complete nucleotide sequences of both viruses can be found in GenBank (Accession Nos. D86068 for HIV_{MCK} and D86069 for HIV_{213}).

RESULTS

Characterization of molecularly cloned HIV_{213} and HIV_{MCK}

Our previous characterization of the biological properties of HIV_{213} and HIV_{MCK}, (Cloyd and Moore, 1990) did not describe the duration of virus production from cells chronically infected with these isolates. Figure 2 shows such analyses, where CEM cells were infected with HIV_{213} or HIV_{MCK} and monitored for p24 expression over time. Approximately 100% of the cells in the cultures became p24 positive and some cells underwent acute viral cytopathicity during the first week postinfection. The cultures recovered and the surviving cells became chronic producers of HIV for a period of time (Fig. 2). The length of time these chronically infected cultures produced virus was significantly different for the two viruses, with the proportion of HIV-producing cells, as judged by p24 immunofluorescence, decreasing over time for both viruses, but HIV_{MCK} decreasing faster than HIV_{213}. At the peak of acute virus replication both virus-infected cultures produced nearly the same amounts of HIV p24 into the culture supernatants (Fig. 3A), and HIV_{213} was more cytopathic than HIV_{MCK}. However, by 5–6 weeks postinfection, only 5% of the HIV_{MCK}-infected cell population still detectably produced HIV p24, while HIV_{213} infected cells required more than 12 weeks to reach the same low level of production. CEM cells chronically infected with the molecular-clones of HIV_{213} and HIV_{MCK} showed the same patterns of shutdown as that of the parental isolates (see Fig. 4), demonstrating that this biological property remained intact.

Duration of chronic expression of chimeric viruses

Rate of shutdown of chimeric viruses possessing substitutions in vif through env regions of genome. To identify the HIV gene(s) influencing differential rates that chronically infected cells shut down virus production, we tested recombinant viruses made between HIV_{213} and HIV_{MCK}. CEM cells were chronically infected with each of the hybrid viruses, and production of virus was monitored by p24 immunofluorescence until the frequency of p24-positive cells diminished to approximately 5% of the cell population. Culture supernatants were also collected and stored at −70° for antigen-capture EIA. Depending on the regions of the genome substituted, the chimeric viruses showed clearly different rates of shutting down.

CEM cells chronically infected with an HIV construct (HIV_{y004}) containing a 2.6-kb fragment of HIV_{MCK} encompassing part of *vpr,* the first exons of *tat* and *rev, vpu,* and 5′ two thirds of *env* in the HIV_{213} genomic background shut down virus production at a slightly faster rate than HIV_{213} (Fig. 4). This rate was not as fast as parental MCK virus, but was intermediate between 213 and MCK. With deletion analysis of the substituted HIV_{MCK} fragment, a 615-bp region alone (HIV_{31}) was shown to be responsible for the rate change (Fig. 4). This *Sal*I–*Nde*I fragment of HIV_{MCK} possessed 62 base pairs of *vpr,* first exons of *tat* and *rev,* and the *vpu* gene. Constructs containing HIV_{MCK} *env* sequences (HIV_4, HIV_{30},

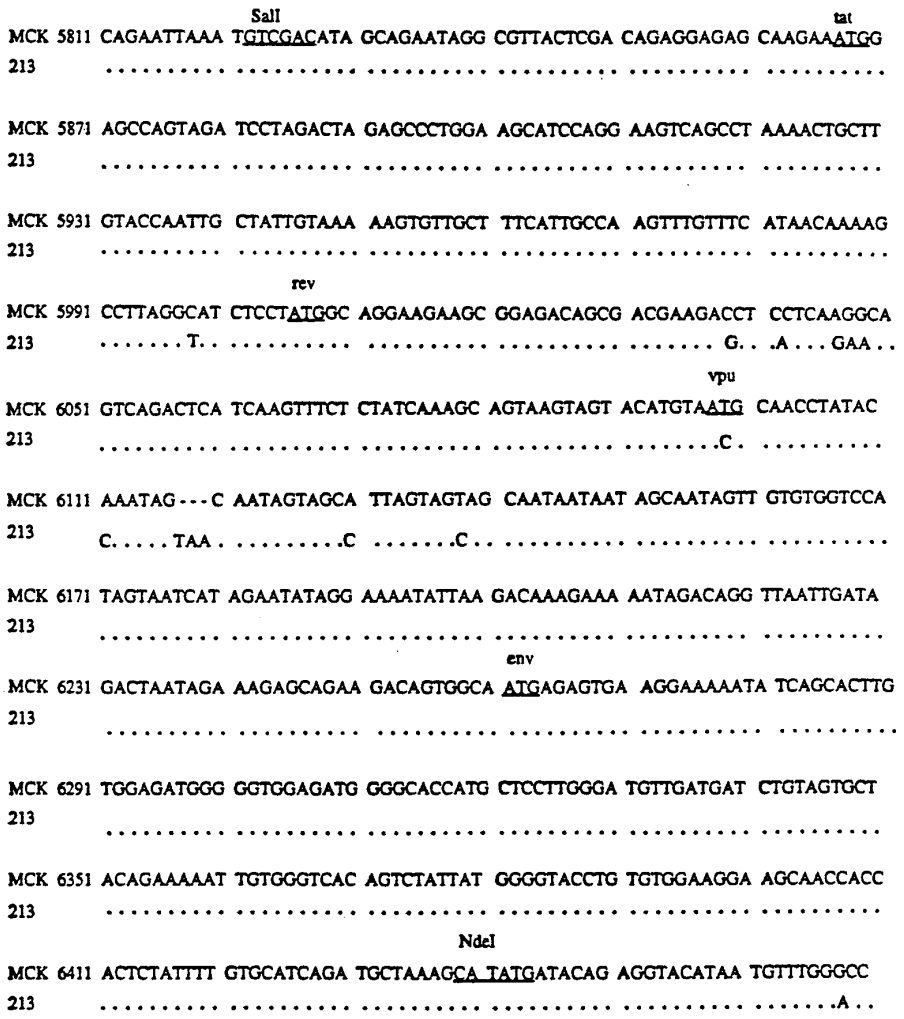

FIG. 5. Sequences of *tat/rev/vpu* region. The nucleic acid sequence was determined by the dideoxy method using the Sequenase kit. Both strands were primed and sequenced. Autoradiograms were read on an IBI gel reader and the sequences were analyzed using the Pustell program.

HIV_{27}, and HIV_{17}) did not demonstrate altered shutdown kinetics from that of HIV_{213} (Fig. 4), demonstrating that the *env* genes of these two viruses were not influencing the differential rate of shutdown.

The *vif* genes of these viruses did not influence this property as demonstrated by construct HIV_{12} (see Fig. 1) which behaved like HIV_{213} (data not shown), nor did HIV constructs $HIV_{24, 14, 25}$, which possessed subregions within *vif* (data not shown, but see Fig. 1 for summary).

Based on sequencing of the *tat/rev/vpu* region (Fig. 5), no nucleotide differences between HIV_{MCK} and HIV_{213} were detected in the *vpr* gene. However, in an overlapping region of *tat* and *rev,* disparate nucleotide sequences between the two viruses were clustered: 5 of 11 base pairs were different (Fig. 5). Amino acids deduced from the nucleic acid sequences were significantly different from each other showing that the Tat and Rev proteins of HIV_{213} and HIV_{MCK} were quite different (Table 1). Furthermore, the translational start codon of *vpu* of HIV_{213} was mutated to ACG. We subsequently found that HIV_{213} does not produce Vpu proteins, (data not shown). Consequently, any one or combination of these three genes (*tat, rev, vpu*) may be slightly influencing the duration of productivity of chronically infected cells.

LTR region profoundly influenced duration of HIV pro-

TABLE 1

Deduced Amino Acid Differences

	TAT			
HIV_{MCK}	Pro_{58}	Pro_{59}	Gly_{61}	
HIV_{213}	Ala_{58}	His_{59}	Asn_{61}	
	REV			
HIV_{MCK}	Asp_{11}	Lev_{13}	Lys_{14}	Ala_{15}
HIV_{213}	Glu_{11}	Ile_{13}	Arg_{14}	Thr_{15}
HIV_{MCK}	Asp_{86}	Ile_{109}	Ala_{114}	
HIV_{213}	Asn_{86}	Val_{109}	Thr_{114}	

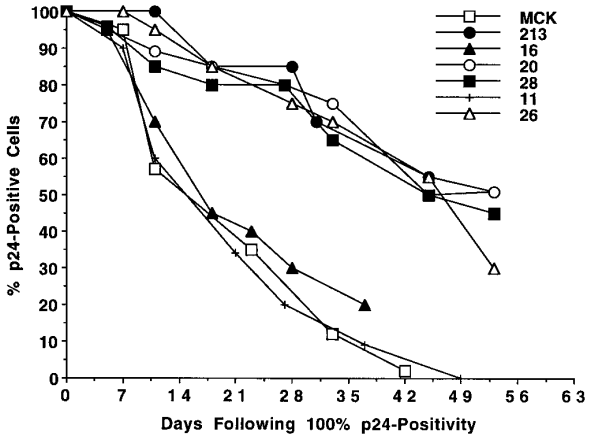

FIG. 6. Kinetics of expression of parental and chimeric viruses containing LTR and *nef* substitutions. The percentage of p24-expressing cells in the infected cultures were determined by fixed-cell immunofluorescence.

duction but nef was not involved. As shown in Fig. 1, construct HIV_{11} consisted of the HIV_{213} genome with its *Xho*I–*Bss*HII fragment derived from HIV_{MCK}. This 1-kb HIV_{MCK} fragment containing *nef* and LTR converted the rate of virus shutdown from the slow kinetics of 213 to the fast kinetics of MCK (Fig. 6). To assess whether *nef* alone was responsible for this effect, a construct (HIV_{20}) possessing the *Bam*HI–*Sca*I fragment of HIV_{MCK} which contains all of *nef,* except for 17 base pairs from the 3′ end, was tested (Fig. 6). Cells chronically infected with this chimeric virus demonstrated a slow rate of shutting down, similar to that of HIV_{213}, and illustrated that the HIV_{MCK} *nef* did not influence this property. The possibility that the HIV_{MCK} 3′ *env* sequences in HIV_{20} might have influenced this phenotype was ruled out with construct HIV_{28}. Together, these data indicated that *nef* was not involved in the differential rate of shutdown of these two viruses. Also, HIV_{16}, that was similar to HIV_{11} but possessed a 223 bp smaller *nef,* showed similar shutdown kinetics to that of HIV_{11} (Fig. 6). This further demonstrated the lack of influence of *nef.* Finally, examining the effect of sequences downstream of the *Bss*HII site, which is 27 nucleotides upstream of the packing signal in the untranslated region before *gag,* and halfway into *gag* (HIV_{26}), resulted in no change in shutdown kinetics. Therefore, it appears that a 3′ 394-bp region in HIV_{MCK} LTR was able to influence the rate of virus shutdown in chronically infected cells.

Sequencing the LTRs from both HIV_{MCK} and HIV_{213} (Fig. 7) revealed that they differed in only five randomly spaced nucleotides. Within the 394-bp 3′ region, there were two nucleotide differences in the interferon-γ (IFN-γ) inducing factor binding site, one 6 bp upstream of the TATA box and one difference in the Tar stem structure. One or more of these nucleotides thus affected the rate of HIV shutdown.

DISCUSSION

Using molecular clones of two HIV isolates which produce virus for significantly different lengths of time following acute infection, we identified the genomic regions which determine the differential rates that these two viruses shut down. A chimeric virus (HIV_{11}) containing the 900-bp *nef*-LTR fragment from HIV_{MCK} rapidly shut down progeny production similar to HIV_{MCK} (see Fig. 1 for summary). Suspecting that *nef* was involved, this region was dissected further (HIV_{20} and HIV_{28}) and it appeared that HIV_{MCK} *nef* was not influencing this property. Some early studies have indicated that *nef* exerts a negative regulatory activity on viral replication or viral transcription, while other studies indicate it does not (Bachelerie *et al.,* 1990; Hammes *et al.,* 1989; Hammes *et al.,* 1989; Kim *et al.,* 1989). More recent studies revealed that *nef* confers enhanced replication to HIV in normal PBLs (Miller *et al.,* 1994; Spina *et al.,* 1994). The 3′ 394 bp of the LTR responsible for accelerated shutdown include the enhancers and the Tat responsive element (TAR). Sequencing of that region has revealed that two nucleotide differences were present in a site where a factor induced by IFN-γ binds, and there were three other scattered single base differences, one of which was in the stem of TAR. The function of IFN-γ inducible factor site in HIV replication is not well understood, while TAT binding to TAR is known to be essential for HIV replication. TAT binds to TAR in a sequence-specific manner at the site of the RNA bulge-loop and thereby facilitates transcription (Dingwall *et al.,* 1989; Drysdale and Pavlakis, 1991). The nucleotide difference in HIV_{213} TAR is localized in the middle of the stem of the TAR RNA loop. It has been shown that mutations in either the bulge-loop or the terminal loop of TAR dramatically affect the level of transactivation by TAT (Lu *et al.,* 1991; Luciw *et al.,* 1987), and the TAR stem has been shown to bind a cellular factor (Marciniak *et al.,* 1990). However, the effect of the nucleotide difference in the TAR stem on viral replication of these viruses is not known. It has been reported that HIV TAR and TAT can substitute for a 5′ splice site, and the stem region of the TAR structure is significantly complementary to U1 snRNA (Lu *et al.,* 1991). It is possible that the nucleotide difference may have physiological significance in that it effects splicing. It is also interesting to note that although these two viruses have very different biological properties, they have very few sequence differences. This shows that minor sequence differences can have significant biological effects. Further studies are in progress to define how this region effects the rate of HIV shutdown during chronic infections.

Independently, the *tat/rev/vpu* region also influenced the rate that chronically infected cells shut down HIV production, but this effect was not as profound as that of the LTR. *Tat* and *rev* are regulatory genes expressed

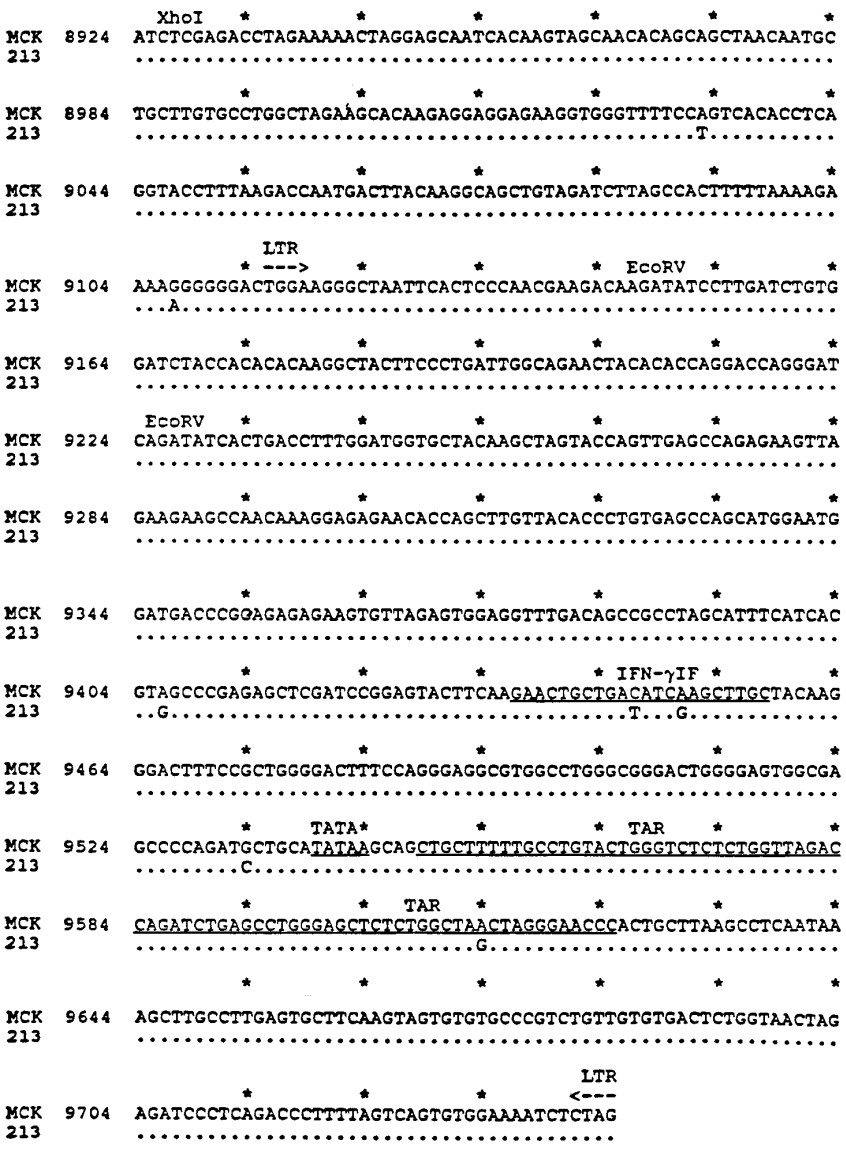

FIG. 7. LTR sequences of HIV_{213} and HIV_{MCK}.

early in the viral replication cycle. The Tat trans-activator serves to increase dramatically HIV LTR-directed transcription (Fisher *et al.,* 1986; Sodroski *et al.,* 1985) via binding to the LTR-encoded TAR element of the nascent mRNA located downstream of the transcription start site (Drysdale and Pavalkis, 1991). The Rev protein binds to RRE in full-length and *env* mRNA, and it is absolutely required for cytoplasmic expression of unspliced and single-spliced HIV RNA species (Felber *et al.,* 1989; Malim and Cullen, 1991; Malim *et al.,* 1989). The Rev protein facilitates the nuclear export of these viral RNA species to the cytoplasm (Chang and Sharp, 1989; Felber *et al.,* 1989; Schwartz *et al.,* 1992). The two viruses were significantly different in an area comprising the first exon of *tat* and *rev,* and the deduced amino acid sequences of those were remarkably different. This suggests the possibility that the activities of the *tat* and *rev* products from the two viruses might be different when combined with any given TAR or RRE. Thus, the possible differences in activities of those proteins may influence the different rates of shutdown of these two viruses.

Although the function of Vpu is not entirely clear, it has been suggested that Vpu may induce disruption of gp160-CD4 complexes. This in turn may increase virion release (Rosen *et al.,* 1988; Willey *et al.,* 1992). Our sequencing data showed that the translation start codon of HIV_{213} *vpu* was mutated to ACG, and we found that HIV_{213} does not produce Vpu protein. It is possible that Vpu then could also affect the length of time HIV progeny is produced during chronic infections. Further work is in progress to define which protein(s) are responsible and how they function.

The results of this study highlighting the LTR as determining fast or slow rates of HIV shutdown during chronic infection are different from the results of Mustafa and Robinson (1993), which showed that a number of the auxiliary genes of the virus play a role in determining how long HIV expresses. The two results actually are not incompatible. Ours showed that with two viruses that are very similar in nucleotide sequence but do shut down at significantly different rates, the major determinant is in the LTR, whereas with the two viruses that Mustafa and Robinson used, it is possible that the relevant LTR sequences were the same and the effect of the auxiliary genes became predominant. Similar to their study, we showed that the *tat/rev/vpu* region also played some role, but it just was not as profound as the LTR. Our study was probably fortunate in that the virus which shut down fast was the noncytopathic virus and the one that shut down slowly was cytopathic. It was just the opposite in the Mustafa and Robinson study, which made their results more difficult to interpret. We were thus able to dissociate cytopathicity from the duration of progeny production. It is likely that a number of HIV genes may interplay and that the true understanding of viral gene function may sometimes require examining the "gestalt" of the virus genome by examining the functions of more than one gene simultaneously.

A final remaining question relates to the importance of differential rates that HIV strains shut down. It is likely that virus which shuts down rapidly escapes the immune response more effectively. On the other hand, a virus which expresses longer would spread the virus more effectively and contribute to increased virus load. We are currently studying this property of HIV strains from long-term "nonprogressor" and "rapid progressor" patients to gain some insight into the potential relevance of differential rates of HIV shutdown.

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