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Replication of Type 1 Human Immunodeficiency Viruses Containing Linker Substitution Mutations in the −201 to −130 Region of the Long Terminal Repeat

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In previous transfection analyses using the chloramphenicol acetyltransferase reporter gene system, we determined that linker substitution (LS) mutations between −201 and −130 (relative to the transcription start site) of the human immunodeficiency virus type 1 long terminal repeat (LTR) caused moderate decreases in LTR transcriptional activity in a T-cell line (S. L. Zeichner, J. Y. H. Kim, and J. C. Alwine, J. Virol. 65:2436–2444, 1991). In order to confirm the significance of this region in the context of viral replication, we constructed several of these LS mutations (−201 to −184, −183 to −166, −165 to −148, and −148 to −130) in proviruses and prepared viral stocks by cocultivation of transfected RD cells with CEMx174 cells. In addition, two mutations between −93 and −76 and between −75 and −58 were utilized, since they affect the nuclear factor κB (NF-κB)- and Sp1-binding sites and were expected to diminish viral replication. Our results suggest that while transfection analyses offer an adequate approximation of the effects of the LS mutations, the analysis of viral replication using a mutant viral stock presents a more accurate picture, which is sometimes at variance with the transfection results. Three mutants (−201/−184 NXS, −165/−148 NXS, and −147/−130 NXS) had effects on viral replication that were much more severe than the effects predicted from their performance in transfection analyses, and the effects of two LS mutations (−201/−184 NXS and −183/−166 NXS) were not predicted by their effects in transfection. In addition, we observed cell type-specific permissiveness to replication of some mutant viruses. In the cell types tested, the LS mutations indicated an apparent requirement not only for the intact NF-κB- and SP1-binding sites but also for several regions between −201 and −130 not previously associated with viral infectivity.

We have previously analyzed the transcriptional activity of the type 1 human immunodeficiency virus (HIV-1) long terminal repeat (LTR) with a set of linker substitution mutants spanning the entire U3 region of the HIV-1 (HXB2) LTR (22–24). These discrete mutations preserve the spatial relationships of the adjacent wild-type LTR sequences. In transient transfection experiments with a chloramphenicol acetyltransferase (CAT) reporter gene system (LTR-CAT construct), we established that transcriptional activation of the LTR involves previously uncharacterized U3 regions and that the functional utility of certain LTR regions is cell type dependent and can vary with cellular stimulation or differentiation (22–24). We have now extended these studies by introducing a selected sample of these mutant LTRs into proviral constructs and analyzing their effects within the context of the viral life cycle.

Generation of wild-type and linker substitution mutant LTR HIV-1 stocks. Linker substitution mutations of the HIV-1 LTR were prepared previously by using polymerase chain reaction technology (23). Each mutant contains a substitution of 18 bp of wild-type HIV-1 (HXB2) LTR sequence with an *Nde*I-*Xho*I-*Sal*I (NXS) polylinker (5′-CATATGCTCG AGGTCGAC-3′). Mutants were labelled according to the *n*/*m* NXS nomenclature, with *n* and *m* referring to the positions of LTR nucleotides substituted by the 5′- and 3′-terminal nucleotides of the NXS polylinker, respectively, relative to the transcription start site. We selected six mutant LTRs for further study. Four linker substitution mutants, which together span nucleotides −201 to −130, altered certain transcription factor-binding sites and, in LTR-CAT transfection studies, displayed between 25 and 70% of the wild-type CAT activity, depending on the mutant (Fig. 1; mutants −147/−130 NXS, −165/−148 NXS, −183/−166 NXS, and −201/−184 NXS). Two other mutants, −75/−58 NXS and −93/−76 NXS, which affect the nuclear factor κB (NF-κB)- and Sp1-binding sites, were selected as controls, since they dramatically decreased LTR-mediated transcription and were expected to demonstrate the most significant effects on replication with mutant viruses (10, 14, 16). Thus, the mutated LTR regions examined in this study were −75 to −58, −93 to −76, −147 to −130, −165 to −148, −183 to −166, and −201 to −184 (Fig. 1).

Each NXS linker substitution LTR was cloned into a plasmid containing a single LTR and complete proviral coding sequences as shown in Fig. 2. The single-LTR proviral fragment was obtained by *Bam*HI digestion of pILIC (a gift from E. Vicenzi, M. Martin, and A. Rabson), which contains complete viral coding sequences originally derived from the infectious molecular clone of HIV-1 (pNL4-3) (10) (Fig. 2A). To avoid the possible complicating effects of mutations in the 3′ LTR on *nef* function, we inserted an *Xba*I linker (Stratagene, San Diego, Calif.), which contains stop codons, into the *nef*-coding region (Fig. 2B).

The resulting plasmid, pG3-ILICX, was also employed in subcloning each single-LTR proviral NXS mutant as well as

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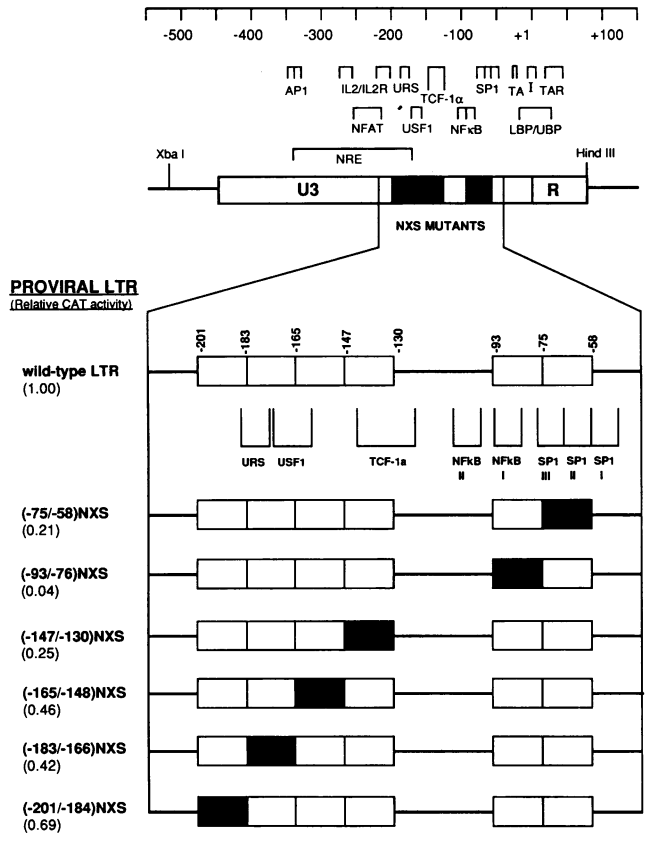

FIG. 1. Map of HIV-1 LTR DNA showing the relative positions of transcription factor-binding sites and linker substitution mutations (3). CAT activity values on the left represent averages relative to wild-type LTR activity from multiple transient transfections of phytohemagglutinin- and phorbol myristate acetate-stimulated Jurkat cells (see text and reference 23). In the expanded diagrams of the mutants, open boxes represent wild-type sequences and filled boxes represent NXS linker substitutions. Abbreviations: AP1, activator protein 1; IL2/IL2R, interleukin-2–interleukin-2 receptor promoter; TA, TATA element; I, initiator element; TAR, *trans*-acting responsive element; LBP/UBP, leader or untranslated binding protein. Nucleotide positions are numbered relative to the transcription start site (+1).

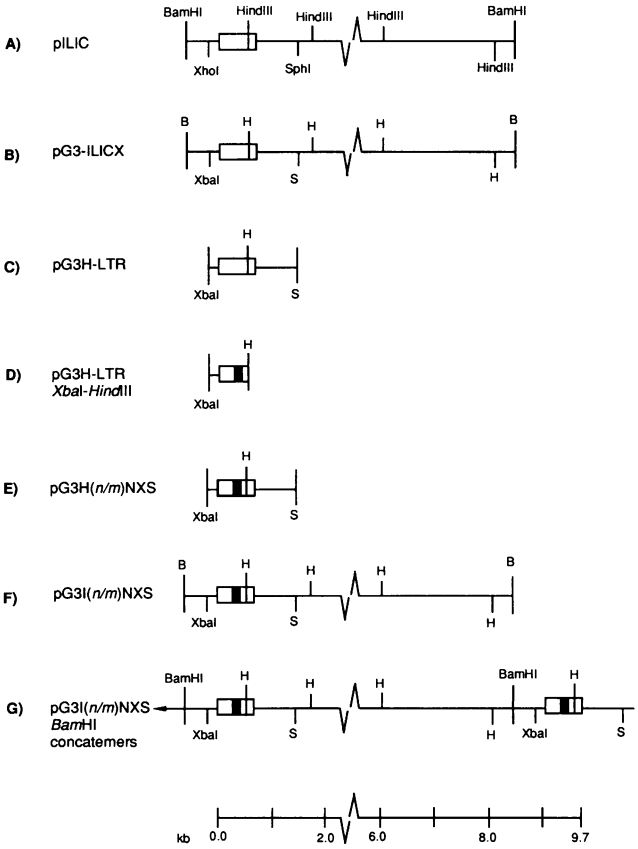

FIG. 2. Construction of linker substitution mutant LTR-containing proviruses (see text). The wild-type NL4-3 proviral fragment with a single LTR (open rectangle) and an intact *Xho*I site was obtained by *Bam*HI digestion of pILIC (A) (10). pG3-ILICX (B), containing a mutation of the *Xho*I site produced by insertion of an *Xba*I linker containing stop codons in each reading frame of *nef*, was digested to release the *Xba*I-*Sph*I LTR-containing fragment, which was cloned into pG3H-LTR (C) for exchange of LTRs. The *Xba*I-*Hin*dIII LTR fragment from linker substitution mutant plasmids, containing the NXS linker (filled box), replaced corresponding wild-type LTR sequences in pG3H-LTR (D) to generate the pG3H *n/m* NXS series (E). The *Xba*I-*Sph*I fragment of pG3H *n/m* NXS replaced the wild-type LTR fragment in pG3-ILICX to generate the pG3I *n/m* NXS series (F). For transfection-cocultivation experiments, concatemers of *Bam*HI proviral fragments of pG3I *n/m* NXS and pG3-ILICX (pG3IWT [wild type]) were generated, restoring LTRs to appropriate 5′ and 3′ positions relative to NL4-3 coding sequences (G). A kilobase scale is shown at the bottom. Abbreviations: B, *Bam*HI; H, *Hin*dIII; S, *Sph*I.

equivalent wild-type sequences from HXB2, the parent HIV strain used for construction of the NXS linker substitution mutants (23). The *Xba*I-*Sph*I LTR-containing fragment of pG3-ILICX was ligated into pGEM3Zf(−) with its *Hin*dIII site deleted (Fig. 2C). The insert was digested with *Xba*I and *Hin*dIII, and the resulting wild-type LTR fragment was replaced by each *Xba*I-*Hin*dIII NXS mutant LTR fragment (Fig. 2D). Each resulting mutant pG3H *n/m* NXS clone was digested with *Xba*I and *Sph*I to release complete mutant LTR inserts (Fig. 2E) for replacement of the LTR of pG3-ILICX to construct a panel of circularly permuted proviral clones, each with a single mutant LTR, called pG3I *n/m* NXS or pG3IWT (Fig. 2F).

Mutant and wild-type HIV-1 infectious DNAs were generated as concatemers of the single-LTR-containing proviral fragment, as previously described (Fig. 2G) (10). Concatemerized proviral DNA (10 μg) was transfected into the RD cell line, a CD4-negative rhabdomyosarcoma-derived human cell line (18), by using Lipofectin (25 μg) in Opti-MEM (both from GIBCO-BRL, Gaithersburg, Md.). Within 48 to 72 h, the transfected RD cultures were cocultivated with 10^6 CEMx174 cells (a T-cell–B-cell hybrid [7, 17]) for an additional 48 to 72 h. Infected CEMx174 cultures were then expanded for 4 to 23 days. This approach proved to be efficient for generating mutant virus stocks, and under cocultivation conditions all six mutant viruses were able to replicate to levels resulting in detectable cytopathic effects (CPE), primarily syncytium formation. To avoid degradation of viral infectivity due to prolonged cocultivation, the more rapidly replicating viruses were harvested earlier than those with lower levels of cytopathic effect. After the viruses were harvested, virus stock titers were standardized by measuring $p24^{gag}$ concentrations with an antigen capture assay (sensi-

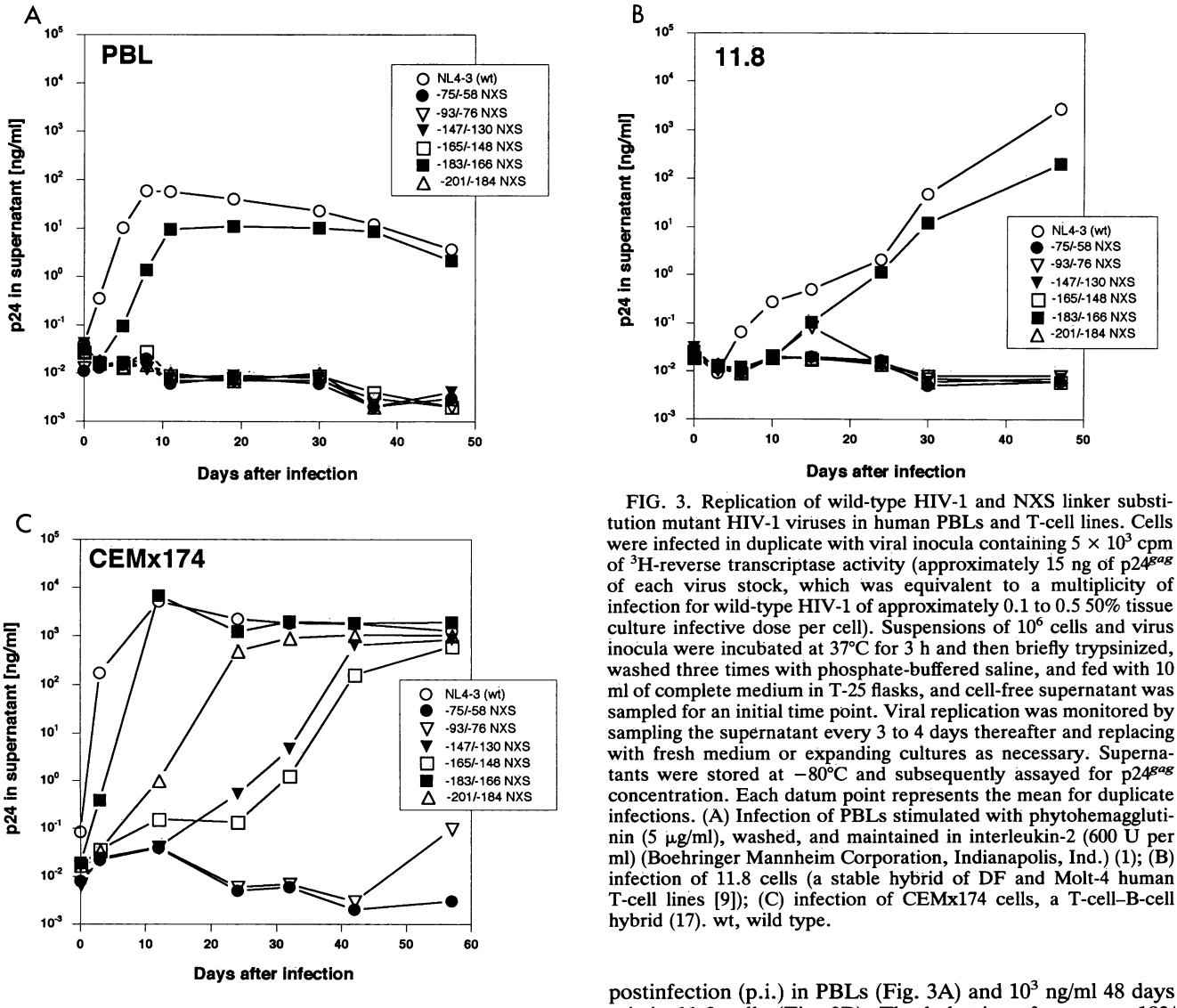

FIG. 3. Replication of wild-type HIV-1 and NXS linker substitution mutant HIV-1 viruses in human PBLs and T-cell lines. Cells were infected in duplicate with viral inocula containing 5×10^3 cpm of ^{3}H-reverse transcriptase activity (approximately 15 ng of $p24^{gag}$ of each virus stock, which was equivalent to a multiplicity of infection for wild-type HIV-1 of approximately 0.1 to 0.5 50% tissue culture infective dose per cell). Suspensions of 10^6 cells and virus inocula were incubated at 37°C for 3 h and then briefly trypsinized, washed three times with phosphate-buffered saline, and fed with 10 ml of complete medium in T-25 flasks, and cell-free supernatant was sampled for an initial time point. Viral replication was monitored by sampling the supernatant every 3 to 4 days thereafter and replacing with fresh medium or expanding cultures as necessary. Supernatants were stored at −80°C and subsequently assayed for $p24^{gag}$ concentration. Each datum point represents the mean for duplicate infections. (A) Infection of PBLs stimulated with phytohemagglutinin (5 μg/ml), washed, and maintained in interleukin-2 (600 U per ml) (Boehringer Mannheim Corporation, Indianapolis, Ind.) (1); (B) infection of 11.8 cells (a stable hybrid of DF and Molt-4 human T-cell lines [9]); (C) infection of CEMx174 cells, a T-cell–B-cell hybrid (17). wt, wild type.

tivity, 10 to 20 pg/ml) and confirmed by determining the reverse transcriptase activity of virus pellets. All virus stocks contained significant amounts of both, with similar reverse transcriptase-to-$p24^{gag}$ ratios. Because of the differences in the titers of some mutant viruses, we were able to attain a maximum equivalent inoculum in subsequent individual-cell-type infections of approximately 15 ng of $p24^{gag}$ per ml. This amount of $p24^{gag}$ resulted in an infectivity of wild-type HIV-1 stocks in CEMx174 cells of 10^5 to 10^6 50% tissue culture infective doses per ml, corresponding to a multiplicity of infection of 0.1 to 0.5 50% tissue culture infective dose per cell.

Infections demonstrate cell type-specific permissiveness. Equivalent inocula of wild-type and mutant virus stocks were used to infect individual cell types, and the production of viral antigen in the supernatant was quantified by the level of $p24^{gag}$ antigen. The results were reproducible among separate infections; typical results are shown in Fig. 3. Stimulated peripheral blood lymphocytes (PBLs) and 11.8 cells (9) supported the replication of only wild-type and mutant −183/−166 NXS viruses. Wild-type HIV-1 achieved peak $p24^{gag}$ levels of approximately 10^2 ng/ml 10 days postinfection (p.i.) in PBLs (Fig. 3A) and 10^3 ng/ml 48 days p.i. in 11.8 cells (Fig. 3B). The behavior of mutant −183/−166 NXS was closest to that of the wild-type virus but demonstrated slower kinetics than the wild type and occasionally produced lower concentrations of viral antigen. In PBL infections, −183/−166 NXS yielded to 10 ng of $p24^{gag}$ per ml 10 days p.i. and 10^2 ng of $p24^{gag}$ per ml 50 days p.i. in 11.8 cells (Fig. 3B). None of the other mutant viruses produced significant levels of $p24^{gag}$ in either of these differentiated T-cell types.

Not surprisingly, the cell line used to raise viral stocks in cocultures, CEMx174, demonstrated the greatest permissiveness for virus replication in single-cell-type infections, achieving $p24^{gag}$ levels of 10^3 ng/ml by 12 days p.i. for both the wild type and mutant −183/−166 NXS (Fig. 3C). CEMx174 was the only cell line in which mutant viruses −147/−130 NXS, −165/−148 NXS, and −201/−184 NXS replicated in sufficient amounts to detect antigen in the supernatant (see below). Mutants −93/−76 NXS and −75/−58 NXS failed to replicate in any of the individual-cell-type infections.

To exclude the possibility of reversion to a wild-type phenotype as an explanation for the growth of −183/−166 NXS and for the observed delayed kinetics in CEMx174

infections with mutants −165/−148 NXS, −147/−130 NXS, and −201/−184 NXS, reinfections of CEMx174 were performed with mutant virus stocks obtained late in productive infections of CEMx174 cells (i.e., up to 60 days p.i.). These reinfections yielded replication kinetics indistinguishable from those of infections with the parental mutant stocks, supporting the validity of the initial observations (data not shown).

In order to ensure that each mutant virus was capable of entry and reverse transcription, polymerase chain reaction was performed on DNA samples obtained from cells infected with DNase I-treated virus stocks. We extracted high-molecular-weight nuclear DNA from 10^6 PBLs at 3 and 30 days p.i. and amplified an HIV-1-specific 115-bp target *gag* sequence using commercial oligonucleotide primers [SK38 (1551/1578); Perkin-Elmer Corporation, Norwalk, Conn.]. The presence of HIV-1 sequences in each sample, detected by ethidium bromide staining in agarose gels (data not shown), confirmed that the observed phenotypes of mutant viruses were due neither to an inability to infect the target cells nor to defects in viral entry and integration. Infected cell DNA preparations were also analyzed by polymerase chain reaction using a primer pair that amplified 577 bp of the LTR sequence (−439 to +138). Each mutant LTR fragment was sequenced, confirming not only the presence of the NXS linkers but also the absence of any additional potentially compensatory LTR mutations (data not shown).

On the basis of their growth characteristics in PBLs and 11.8 and CEMx174 cells, the six mutant viruses were classified into three broad phenotypes.

(i) Wild-type replication phenotype in all cell types. Mutant −183/−166 NXS consistently demonstrated a replication phenotype similar to that of the wild type in all three cell types. The mutant linker substitutes 8 bp of the possible upstream repression sequence (URS) (−188 to −176) and 8 bp of the binding site of upstream stimulatory factor 1 (USF-1) (−173 to −160), a putative negative regulatory protein (Fig. 1) (2, 12). Hence, mutations in nucleotides −183 to −166 alone do not produce a phenotype indicative of a negative regulatory function for this region. These infection results contrast with our previously reported transfection experiments with Jurkat cells. In those experiments, moderately decreased LTR-CAT expression (42% of that for the wild type) (Fig. 1) was observed (23).

(ii) Severely reduced replication phenotype in all cell types. Linker substitution mutation of NF-κB- and Sp1-binding sites caused drastic diminution of viral replication in all three cell types (Fig. 3). Mutant −93/−76 NXS substitutes the entire 3′-proximal NF-κB-binding site, NF-κB-I (−91 to −81), and 3 bp of the 5′-proximal Sp1-binding site, Sp1-III (−78 to −67) (Fig. 1). Mutant −75/−58 NXS replaces most of both the 5′-proximal (Sp1-III [−78 to −67]) and the middle (Sp1-II [−66 to −57]) Sp1-binding sites (Fig. 1). Their dramatic effects in viral infections agree with our previous transfection analysis, in which these mutations had the greatest effects on LTR-CAT expression, resulting in as little as 4 and 21% of the wild-type expression, respectively (Fig. 1) (23). Our data illustrate the absolute requirement of these *cis*-acting elements for efficient viral replication in these cell types.

(iii) Severely reduced replication phenotype in PBLs and 11.8 cells but delayed replication kinetics in CEMx174. Although PBLs and 11.8 cells failed to support infections with viruses −201/−184 NXS, −165/−148 NXS, and −147/−130 NXS, these viruses replicated successfully in the highly permissive CEMx174 cells, both in the initial transfection-cocultivation and in the subsequent individual-cell-type infections at lower multiplicities of infection (Fig. 3C). Since no significant replication of these mutants in either PBLs or the 11.8 cells was observed, the substituted regions appear necessary for growth in these differentiated T-cell types. The regions altered by these mutants are poorly characterized with respect to transcriptional regulation. Mutant −165/−148 NXS contains a substitution of the downstream 6 bp of the USF-1-binding site and exhibited delayed kinetics in CEMx174 infection but produced levels of $p24^{gag}$ (10^3 ng/ml) close to those for the wild type by 42 days p.i. Virus −201/−184 NXS also produced wild-type concentrations of $p24^{gag}$ 25 days p.i., despite the substitution of potential negative regulatory element (NRE) sequences. Mutant −147/−130 NXS substitutes 10 bp of the T-cell factor 1α–lymphoid enhancer-binding factor 1 (TCF-1α/LEF-1)-binding site (−139 to −124) and, like mutant −165/−148 NXS, attained $p24^{gag}$ levels of 10^3 ng/ml 40 days p.i. in CEMx174 infections (Fig. 3C).

Transcription factor-binding sites affected by the linker substitution mutations in the context of viral replication. (i) NF-κB and Sp1. Our results establish the absolute requirement of NF-κB- and Sp1-binding sites for viral replication under the conditions examined. These findings are in agreement with reports that conclude that Sp1 sites are critical for HIV-1 replication (5, 8) but contrast with observations that intact NF-κB- and Sp1-binding sites, in various combinations, were dispensable for viral replication under certain conditions (10, 14, 16). The differences in results may be due to the levels of NF-κB and Sp1 activities under the different conditions and with the different cell types examined.

(ii) USF-1 and URS. All three cell types were permissive for replication of −183/−166 NXS, with little difference in kinetics or output compared with the wild type. As noted above, this contrasts with our earlier transfection findings and does not support the role of USF-1 in negative regulatory activity (12). The linker substitution in mutant −201/−184 NXS replaces sequences between the nuclear factor of activated T cells (NFAT)-binding site and the URS, previously suggested to possess negative regulatory activity (4, 11, 13, 15). Mutations in the putative NRE might be expected to have either no effect or increased replication kinetics. Mutant −201/−184 NXS replicated only in CEMx174 cells and with delayed kinetics. These data suggest that the sequences from −201 to −184 have a positive rather than negative regulatory function.

(iii) TCF-1α/LEF-1. Another mutant virus which replicated only in CEMx174 cells, −147/−130 NXS, contains a substitution of the 5′-most 10 bp of the TCF-1α/LEF-1-binding site. Its delayed growth rate in CEMx174 cells and its inability to replicate in PBLs and 11.8 cells suggest that this binding site may play a significant role in viral transcription (Fig. 3). TCF-1α/LEF-1 is a thymus- and lymphocyte-specific factor which activates transcription via binding to its pyrimidine-rich target sequence (6, 19, 20). Its transcriptional activity appears to be dependent upon the sequence context of the binding site, which may explain the negative effects of linker substitution mutations surrounding the −147/−130 NXS mutation (21).

Summary. In a previous transient-transfection analysis using T-cell lines, we determined that linker substitution mutations in the LTR region between −201 and −130 lowered transcription to between 25 and 75% of that for the wild-type LTR, depending on the position of the linker (23). These LTR sequences had not been previously associated with transcriptional activity. By incorporating these LTRs

into proviruses, we have obtained results indicating that infections with mutant virus stocks demonstrate a different, possibly more accurate, analysis of the effects of these linker substitution mutations. For example, mutants −147/−130 NXS and −165/−148 NXS demonstrated effects on viral replication that were more severe than the effects predicted by their transfection results, whereas the effect of mutant −183/−166 NXS was less severe than expected (23).

We have also observed that the replication phenotype of linker substitution mutant viruses depended on the host cell type infected. In general, the more differentiated T-cell types, PBLs and 11.8 cells, were much less permissive for replication of the mutant viruses than CEMx174 cells were (CEMx174 cells > 11.8 cells = PBLs). This hierarchy of cell type dependence of viral replication suggests that the HIV-1 LTR may influence cell tropism and that some cell types are more permissive for LTR utilization.

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