Virology 440 (2013) 31–40

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

# Mutational analysis of the internal membrane proximal domain of the HIV glycoprotein C-terminus

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ARTICLE INFO

*Article history:*
Received 19 December 2012
Accepted 31 January 2013
Available online 5 March 2013

*Keywords:*
HIV Env C-terminus
Conserved region
Env incorporation
Rev-independent Env expression
HIV infection

ABSTRACT

This study focuses on the long stretch of highly conserved amino acids in the membrane proximal part of the HIV-1 cytoplasmic tail (Env amino acids (aa) 706–718) upstream of the overlap with the *tat* and *rev* second coding exons. Changes in Env aa 713 and 715, although they did not affect Env function, abrogated replicative spread. Other amino acid substitutions, i.e., 706–712, 714 and 716, despite their conservation, did not result in defective replicative phenotypes even in primary peripheral blood lymphocytes. Our results point to their involvement in presently unrecognized essential Env functions pertinent only in *in vivo*. Interestingly, changes in the codons for residues 717–718 as well as some mutations in residues 714–716 abrogated Gag expression but still allowed expression of functional Env in a *rev*-independent manner. This could be due to the inactivation of a rev-regulated negative element within the respective nucleotide sequence (8354–8368).



## Introduction

A conserved feature of most lentiviral glycoproteins, including the Env glycoprotein of human immunodeficiency virus type 1 (HIV-1), is the presence of a very long cytoplasmic tail region (Env-CT) which, in the case of HIV, is 151 amino acids (aa) long. The properties of the HIV Env-CT and its contributions to essential viral functions have been extensively studied. They are summarized in recent reviews (Checkley et al., 2011; Postler and Desrosiers, 2013) and will only be briefly described here. Thus, the Env-CT, presumably mediated by its interaction with the viral matrix protein (MA), has been proposed to play roles in Env incorporation into virions (Freed and Martin, 1996) and in the regulation of fusion between released virions and the membrane of target cells (Murakami et al., 2004; Wyma et al., 2004). Several structural and functional motifs are located within the region. Most prominent of these are three so-called lentiviral lytic peptide (LLP1-3) motifs located in the central and C-terminal region of the Env-CT. These motifs have been shown to interact with cellular membranes and have been proposed to be involved in multiple Env-mediated functions (for review, see Checkley et al. (2011)). Two functional endocytosis motifs are also located in the Env-CT, namely a membrane-proximal tyrosine-based signal (YxxL) and a C-terminal dileucine motif (Env-LL855,856), both of which operate independently through interaction with clathrin adaptor protein complexes (Berlioz-Torrent et al., 1999; Byland et al., 2007; Wyss et al., 2001). The membrane-proximal YxxL motif has also been shown to play a role in the basolateral targeting of Env (and Gag) in polarised cells (Lodge et al., 1994; Lodge et al., 1997).

Mutational analysis of the Env-CT has been quite extensive and the phenotypes of mutant virions carrying a wide variety of point mutations and internal and C-terminal deletions in the Env-CT have been analysed. Perhaps surprisingly, the Env-CT region is actually dispensable for replication in certain laboratory-derived cell lines but its presence is absolutely essential for replicative viral spread in most T-cell lines and in primary PBMCs (Murakami and Freed, 2000b). The existence of an HIV Env-CT region is extremely highly conserved *in vivo* and, in the simian immunodeficiency virus/monkey model, truncation of the Env-CT led to suppression of viral replication *in vivo* (Shacklett et al., 2000).

The functional roles currently attributed to the long HIV-Env-CT can, however, be fulfilled by the glycoproteins of other retroviruses (apart from lentiviruses), as well by the glycoproteins of enveloped viruses in general, all of which carry much shorter CT regions. This suggests that additional lentiviral Env-CT functions, essential for viral replication and pathogenesis *in vivo*, still remain to be elucidated. One approach, which potentially promised to yield information relevant to this, has been the identification of cellular proteins interacting with the Env-CT. In fact, several confirmed Env-CT interacting cellular proteins have been found

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http://dx.doi.org/10.1016/j.virol.2013.01.025

but, unfortunately, until now have not further helped to elucidate novel Env-CT functions (for review, see Checkley et al. (2011)).

The mutational analyses on the HIV Env-CT carried out to date, have frequently focussed on identifiable structural or functional motifs, e.g., on the LLP1-3 domains or on motifs implicated in endocytosis, sorting or potential post-translational modification (e.g., cysteine palmitylation). One of the longest stretches of highly conserved amino acids within the Env-CT domain is the section of 13 residues immediately proximal to the viral membrane and preceding the overlap with the reading frames for the *rev* and *tat* second coding exons. The presence of the membrane-proximal tyrosine-based endocytosis signal as well as nucleotide sequence restriction required for functional splicing at splice acceptor 7 (SA7) may contribute to this conservation. Nevertheless, since it would appear that neither of these occurrences would require the high conservation observed, we have performed extensive mutational analysis of the region and analysed the phenotypes of the respective mutants with respect to Env function and viral replication.

## Results

### *Properties of mutant virions with alanine replacements*

The first 13 aa (residues 706–718) of the HIV Env cytoplasmic tail (Env-CT), i.e., the region directly following the Env-TMD and thus adjacent to the membrane, represents one of the longest stretches of conserved aa within the Env-CT domain. In Fig. 1A, the sequence of these aa in the Env protein of the HIV-1 strain BH10 (Ratner et al., 1985) is shown and the percentage conservation of each residue is given as evaluated using the Quickalign tool with reference to the 2011 web alignment provided by the HIV Database, Los Alamos, New Mexico, USA (http://www.hiv.lanl.gov/). Ten of 13 residues exhibit >95% conservation, Q710 is 91.5%, N706 is 88.1% and F717 is 82.3% conserved. The percentage conservation of the neighbouring aa (residues 702–705 and 719–721), which were included in this study, is also shown. In order to gain information as to the possible functional roles of the membrane proximal Env-CT region, extensive mutational analysis was performed. In a first set of mutants, Env aa 702–721 were changed in groups of 3 (in one case 2), in all cases to alanine, except in the case of THL719–721 which were mutated to ILH (thus maintaining the aa sequence of the overlapping Rev protein) (Fig. 1A). HEK 293 T cells were transfected with pNL-Wt and the respective mutant constructs. Fig. 1B shows Western blot analysis of cell-associated Env (gp160/gp120 and gp41) and Gag (p24 is shown, its amounts reflects the amounts of expressed intracellular Gag proteins (Pr55gag and cleavage products)). Env proteins were generated in all cases and proteolytically processed to gp120 and gp41. As determined by immunoblot of cell lysates (Fig. 1B) and CA-ELISA of released virions in culture supernatants (not shown), Gag proteins were also generated in equal amounts in all cases except in the cases of pNL-Env-714–716–AAA and pNL-Env-717–718–AA where no p24 was detectable at all. As will be discussed later (Fig. 2), this is due to functional destruction of the splice acceptor 7 (SA7) sequence.

The Wt and all of the mutant Env proteins exhibited cell–cell fusion activities (reflecting the sum of Env cell surface expression level and fusion ability) when expressed in transfected HEK 293 T cells, cocultivated with CD4/coreceptor expressing Tzm-bl reporter cells. The extents of cell–cell fusion were equivalent to Wt in all cases except in the cases of pNL-Env-714–716-AAA and pNL-Env-717–718-AA which were reproducibly reduced to about 50% of Wt (Fig. 1C). This reduction may be due to altered cell surface expression in the absence of coexpressed Gag.

Fig. 1D (top) shows the relative infection levels in Tzm-bl reporter cells of Wt and mutant virions released into the supernatants of transfected HEK 293 T cells. Three of the mutants (pNL-Env-702–704-AAA, pNL-Env-705–707-AAA and pNL-Env-719–721-ILH) exhibited infection levels equal to, or more than, Wt, two mutants (pNL-Env-708–710-AAA, pNL-Env-711-713-AAA) exhibited reduced infection levels and, as to be expected, there was no detectable infection levels in the supernatants lacking expressed Gag protein (pNL-Env-714–716-AAA, pNL-Env-717–718-AA). In Fig. 1D, bottom, the abilities of the same virions to achieve spreading infection in H9 T-cells is shown. To analyse this, cells were infected at very low MOI with equal amounts of cell-free virions and stained with anti-HIV serum on days 1, 2, 4, 6 and 7 p.i.. Virions which resulted in 100% positive H9 T-cells on 7 day p.i. were scored as infectious (+). Virions which are scored as (−−) exhibited no or only a very low percentage of positively stained H9 T-cells on day 7. In general, those mutants with Wt infection levels in Tzm-bl cells achieved spreading infection in H9 T-cells and those lacking, or with clearly reduced, Tzm-bl infection levels could not achieve spreading infection (Fig. 1D). An exception was mutant pNL-Env-708-710-AAA which, despite repeatedly exhibiting reduced Tzm-bl infectivity, was still able to mediate spreading infection in H9 T-cells.

Fig. 1E shows the gp120 content of released Wt and mutant virus particles. Virions were generated in H9 T-cells since it has been reported that Env protein incorporation into HIV particles is cell-dependent (Akari et al., 2000; Murakami and Freed, 2000b). Efficient proviral expression in these cells was achieved by infection with VSV-G pseudotyped Wt and mutant virions produced in HEK 293 T cells (virus production was, however, not possible in the cases of mutants pNL-Env-714–716-AAA and pNL-Env-717–718-AA, which fail to express Gag). Virus particles newly produced in the H9 T-cells were collected and concentrated at 48 h post infection. As can be seen in Fig. 1E, all of the analysed virions incorporated approximately equal amounts of gp120 except pNL-711–713-AAA which exhibited reduced gp120 content.

### *Effect of nucleotide changes affecting splice acceptor 7*

The nucleotide changes in some of the mutants are close to the splice acceptor site (SA7) for the *rev* and *tat* second coding exons (downstream junction at nucleotide 8369 in pNL4-3). Using the MaxEntScan tool (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html), it could be determined that the splicing scores at SA7 in pNL-Env-714–716-AAA and pNL-Env-717–718-AA were reduced to −8.43 and −14.52, respectively, (in comparison to the Wt score of 7.15) whereas there was no significant change in the cases of the other mutants (see summary in Table 2). Note that higher scores are indicative of better splicing potential. In order to analyse SA7 splicing directly, diagnostic fragments spanning the SA7 site were amplified by RT-PCR from HIV mRNAs expressed in transfected HEK 293 T cells. The positions of the employed primers within the HIV genome are shown in Fig. 2A. Two different primer pairs amplifying fragments from 2 kb intronless mRNA species (Fig. 2B, left panel) or from 4 kb intron-containing mRNA species (Fig. 2B, right panel) were employed. The assignment of amplified fragments to specific viral RNA species has been carried out as described (Purcell and Martin, 1993) and these assignments are shown on the right. It can clearly be seen that, in comparison to pNL-Wt, pNL-Env-714–716-AAA and pNL-Env-717–718-AA did not display the normal SA7-dependent splicing pattern but rather exhibited aberrant products. On the other hand, pNL-Env-711–713-AAA, which had a splicing score very close to that of Wt, maintained the Wt splicing pattern. These data strongly point to a splicing defect

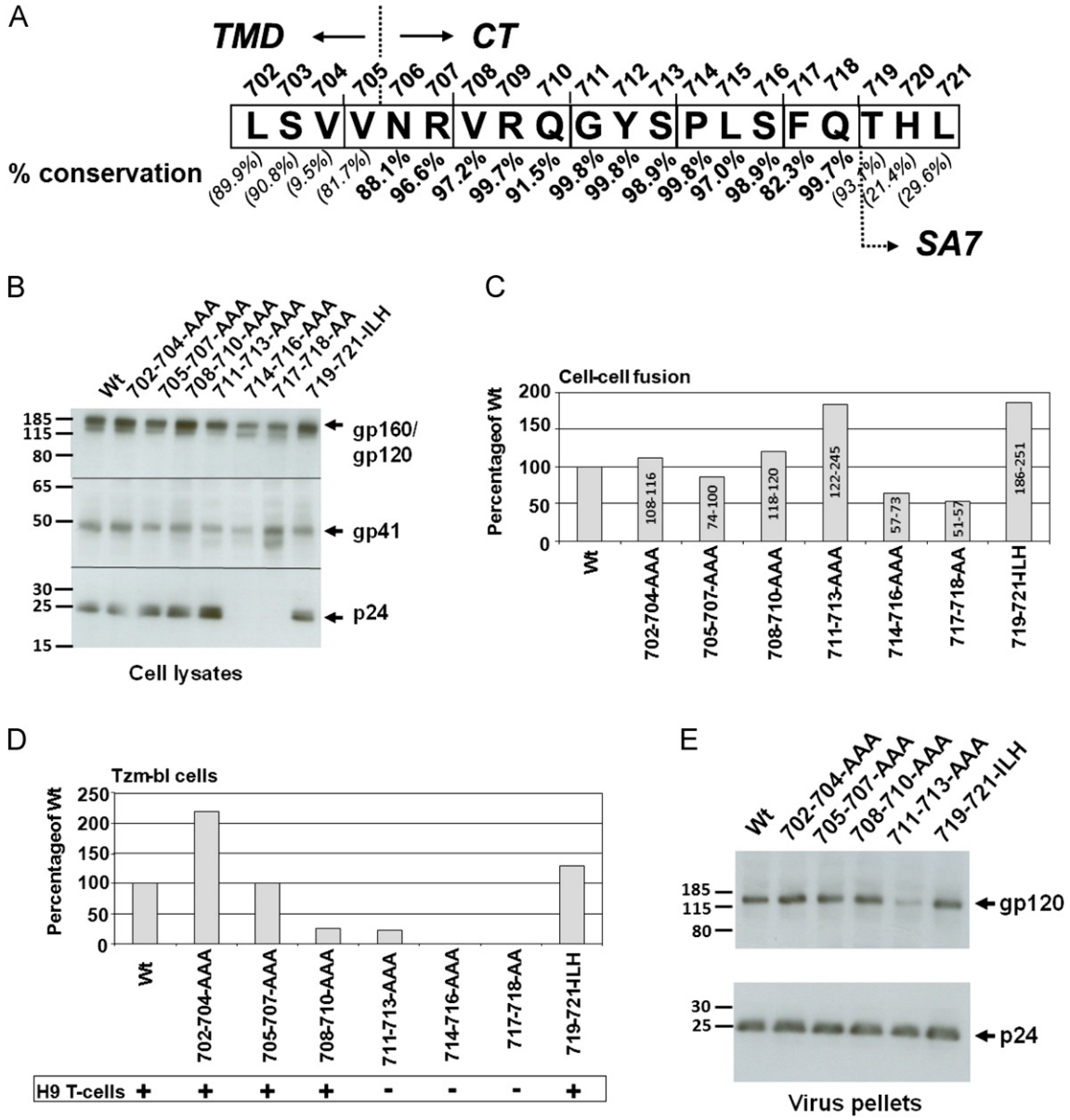


**Fig. 1.** Properties of virions mutated within the internal membrane proximal region of the Env-CT. (A) The sequence of Env residues 702–721 (the major focus has been on aa 706–718) and the percentage conservation of individual residues are shown. The arrows above indicate the border of the Env-TMD and CT domains and the arrow below the border of splice acceptor 7 (SA7). The individually boxed groups of 3 (in one case 2) amino acids have been mutated to alanine (A) (except residues THL719–721 which have been mutated to ILH). (B) Western blot analysis of lysates of HEK 293 T cells transfected with pNL-Wt and the indicated proviral constructs. The top portion of the filter has been probed with anti-gp120 serum, the middle portion with gp41 antibodies (Chessie 8) and the bottom portion with p24 antibodies. (C) Cell–cell fusion activities of mutant glycoproteins as percentage of the activity of pNL-Wt-Env. The average of two independent experiments is shown (the range of values is shown within the respective bars). (D) Top: Infection levels of mutant virions in Tzm-bl indicator cells as percentage of the activity of pNL-Wt. A representative experiment from at least two independent experiments (in the case of pNL-Env-707–710-AAA four independent experiments) is shown. Bottom: abilities to mediate spreading infection in H9 T-cells. +indicates infection similar to pNL-Wt,—indicates absent or only minimal infection. 3 independent experiments were performed. E. Western blot analysis of virions released from infected H9 T-cells. The top portion of the filter has been probed with anti-gp120 serum and the bottom portion with p24 antibodies. In B. and E., the positions of detected HIV proteins are indicated on the right and of molecular weight markers on the left.

and a resulting failure to express functional Rev protein, being the reason for the lack of Gag expression from pNL-Env-714–716-AAA and pNL-Env-717–718-AA. Tat transactivation ability is less likely to be impinged since exon 2 is not essential for this function (Green and Loewenstein, 1988). As shown in Fig. 2C, Gag expression from pNL-Env-714–716-AAA and pNL-Env-717–718-AA could be completely restored when HIV- *rev* (and *tat*) was provided *in trans* by cotransfection with the subviral construct pLTR-ctat/crev. Virion particles were also released into the medium (as established by CA-ELISA) but these were only very poorly infectious in a single-round assay in Tzm-bl cells (approx. 4% of pNL-Wt, data not shown). Thus the data in Fig. 1B and Fig. 2C show that, in the cases of pNL-Env-714–716-AAA and pNL-Env-717–718-AA, Env expression was independent of functional SA7 splicing and thus presumably also of Rev protein expression. To test this directly, we have additionally introduced a stop codon in the *rev* reading frame (leaving the overlapping *env* reading frame open) in the context of pNL-Env-714–716-AAA (and pNL-Wt) and examined Env and Gag expression. As to be expected, in the case of pNL-Wt, lack of functional Rev protein completely abrogated both Env and Gag expression. On the other hand, Env expression from pNL-Env-714–716-AAA was unaffected (Fig. 2D) and was thus occurring independently of expressed Rev protein.

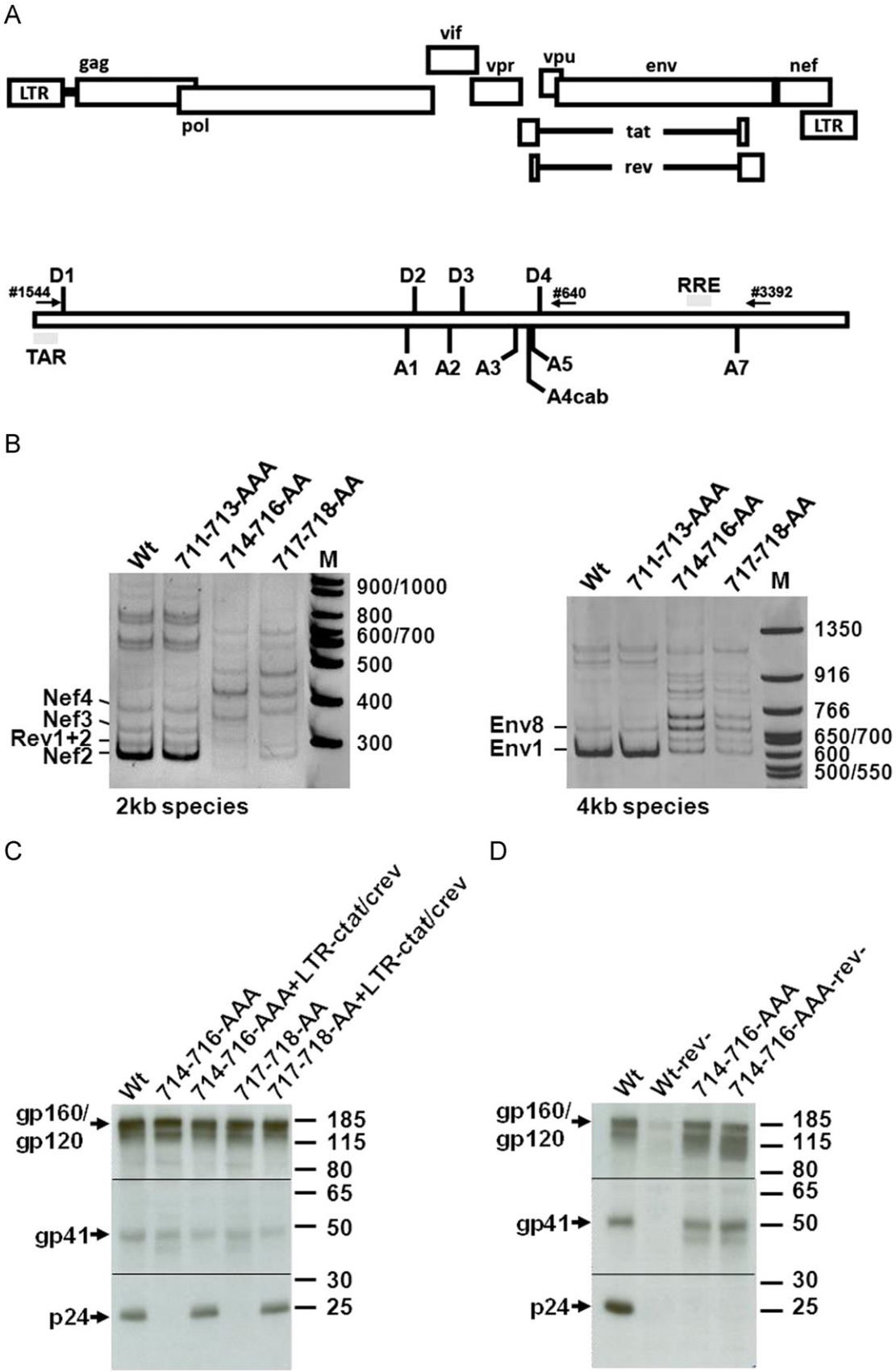


**Fig. 2.** Viral mRNA and protein expression from proviral constructs with abrogated SA7 splicing. (A) Schematic picture of the HIV-1 genome showing the relative positions of the 5′splice sites (ss) (D1 to D4) and 3'ss (A1 to A7) within the viral 9 kb pre-RNA. The positions of the primers (#1544, #640, #3392) used in the RT-PCR analysis are indicated by arrows (primer sequences are given in the material and methods section). LTR (long terminal repeat); RRE (Rev responsive element); TAR (trans-activation response element). (B) RT-PCR amplified fragments from HIV mRNA species in lysates of HEK 293 T cells transfected with the indicated constructs. The primer pair #1544/#3392 was used to amplify the 2 kb intronless mRNA species (left panel) and primer pair #1544/#640 to amplify the 4 kb intron-containing mRNA species (right panel). The RT-PCR products were resolved by non-denaturating polyacrylamide gel electrophoresis and stained with ethidium bromide. DNA fragment size markers are present in lanes M. The assignment of amplified fragments to specific viral RNA species has been carried out as described (Purcell and Martin, 1993) and these assignments are shown on the left. (C) Western blot analysis of lysates of HEK 293 T cells transfected with the indicated plasmids without or with cotransfection of pLTR-ctat/crev as indicated. D. Western blot analysis of lysates of HEK 293 T cells transfected with pNL-Wt and pNL-Env-714–716-AAA without or with an additional mutation in the *rev* gene (expressing truncated Rev-Tr62 protein) as indicated. In both (C and D), the top portions of the filters have been probed with anti-gp120 serum, the middle portions with gp41 antibodies (Chessie 8) and the bottom portions with p24 antibodies. The positions of viral proteins are given on the left and of molecular weight markers on the right.

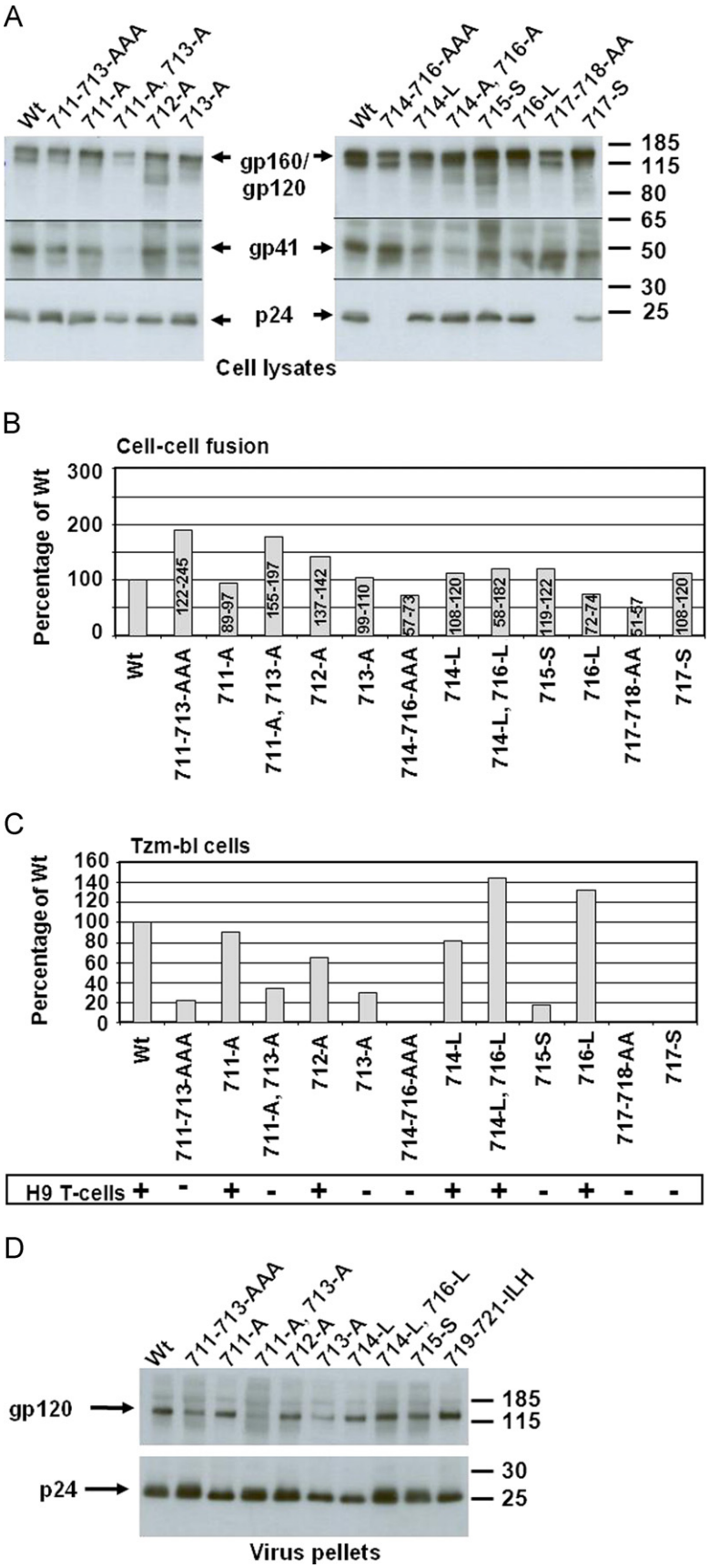


**Fig. 3.** Properties of further mutants with retained splicing abilities. (A) Western blot analysis of lysates of HEK 293 T cells transfected with pNL-Wt and the indicated proviral constructs. The top portions of the filters have been probed with anti-gp120 serum, the middle portions with gp41 antibodies (Chessie 8) and the bottom portions with p24 antibodies. The positions of detected HIV proteins are indicated in the middle and of molecular weight markers on the right. (B) Cell–cell fusion activities of mutant glycoproteins as percentage of the activity of pNL-Wt-Env. The experiment shown is the average of 2 independent experiments (the range of values is shown within the respective bars). (C) Top: Infection levels of mutant virions in Tzm-bl indicator cells as percentage of the activity of pNL-Wt. Bottom: abilities to mediate spreading infection in H9 T-cells. +indicates infection similar to pNL-Wt,—indicates absent or only minimal infection. 3 independent experiments were performed. (D) Western blot analysis of virions released from infected H9 T-cells. The top portion of the filter has been probed with anti-gp120 serum and the bottom portion with p24 antibodies. The positions of detected HIV proteins are indicated on the left and of molecular weight markers on the right.

*Properties of additional mutant virions with functional SA7 splicing*

Three of the mutants studied so far (pNL-Env-711–713-AAA, pNL-Env-714–716-AAA and pNL-Env-717–718-AA) were defective, in two cases as a result of abrogated SA7 splicing. We thus aimed to generate further mutations, which allow assessment of the putative importance of Env aa 711–718 for viral replication independent of effects on SA7 splicing. Table 1 lists the mutations generated and the respective SA7 splicing scores (these again calculated using the MaxEntScan tool (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html). The new mutations within aa 711–716 all had little effect on splicing scores. However, most changes at F717 abrogated splicing and the selected mutation F717S still resulted in a reduced splicing score from 7.15 to 5.58 (Table 1). It was not possible to change residue 718 without negatively affecting SA7 splicing score.

**Table 1**
Mutations within Env aa 711–718.

| Amino acids 711–718 | | | | | | | | Splicing score | Name |
|---|---|---|---|---|---|---|---|---|---|
| **G** 711 | **Y** 712 | **S** 713 | **P** 714 | **L** 715 | **S** 716 | **F** 717 | **Q** 718 | 7.15 | Wt |
| A | A | A | | | | | | 7.10 | 711–713-AAA |
| A | | | | | | | | 7.15 | 711-A |
| A | | A | | | | | | 6.66 | 711-A, 713-A |
| | A | | | | | | | 7.72 | 712-A |
| | | A | | | | | | 6.66 | 713-A |
| | | | A | A | A | | | −8.43 | 714–716-AAA |
| | | | L | | | | | 7.19 | 714-L |
| | | | L | | L | | | 7.05 | 714-L, 716-L |
| | | | | S | | | | 6.51 | 715-S |
| | | | | | L | | | 7.01 | 716-L |
| | | | | | | A | A | −14.52 | 717–718-A |
| | | | | | | S | | 5.58 | 717-S |

The amino acid changes of the respective Env residues, the splice score at SA7 (MaxEntScan tool (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html)) and the names of the generated mutants are given.

The properties of these new mutant virions are shown in Fig. 3. All the new mutant constructs expressed Gag protein as determined by Western blot (Fig. 3A) and CA-ELISA of culture supernatants (not shown) whereas, as before, pNL-Env-714–716-AAA and pNL-Env-717–718-AA failed to do so. The Gag amounts, were equal except in the case of pNL-Env-717-S, which, in accordance with its reduced splicing score, expressed only about one third of the amount of released p24 as pNL-Wt. In fact, direct analysis of SA7 splicing activity of pNL-Env-717-S by RT-PCR of HIV-mRNA again revealed a pattern of products differing from those from pNL-Wt although not as drastically as was the case for pNL-Env-714–716-AAA and pNL-Env-717–718-AA in Fig. 2B (data not shown). All the mutant constructs expressed proteolytically processed Env proteins (Fig. 3A). In general, the mutants expressed amounts of Env approximately equal to that of pNL-Wt. An exception was pNL-Env-711-A, 713-A which in half of the experiments performed (4 experiments) expressed less Env. The reason for this variability is not clear. All of the mutant glycoproteins were functional and able to mediate cell–cell fusion in most cases to levels equal to or higher than Wt-Env (Fig. 3B). Again the two mutant constructs pNL-Env-714–716-AAA and pNL-Env-717–718-AA, which fail to express Gag, exhibited reduced cell–cell fusion activities (to about 50% of Wt-Env). Infection levels in Tzm-bl reporter cells are shown in Fig. 3C, top panel and the abilities of the virions to achieve spreading infection in H9 T-cells in the bottom panel. As before, pNL-Env-714–716-AAA and pNL-Env-717–718-AA, with splicing defects, were non-infectious and this was also the case for pNL-Env-717-S, which has a reduced SA7 splicing score and expresses less Gag protein. The mutants pNL-Env-711–713-AAA, pNL-Env-711-A, 713-A and pNL-Env-713-A, which all share the S713A mutation, exhibited reduced infection levels in Tzm-bl cells and did not mediate spreading infection in H9 T-cells. This was also the case for mutant pNL-Env-715-S. All of the other constructs exhibited levels of infection in Tzm-bl cells, which were equal to pNL-Wt or slightly increased or decreased, respectively, and all of these mutant virions could mediate spreading infection in H9 T-cells. In Fig. 3D, incorporation of gp120 into released virions generated in H9 T-cells is shown. In all cases, gp120 incorporation occurred but was somewhat reduced in the cases of pNL-Env-711–713-AAA, pNL-Env-711-A, 713-A and pNL-Env-713-A, the constructs which share the S713A mutation and are replication-defective in H9 T-cells.

In order to analyse viral replication in a more relevant system and to more closely follow replication kinetics, freshly prepared activated human PBMCs were infected with equal amounts (as determined by CA-ELISA) of Wt and mutant virions. After removal of input virions and washing, the amounts of newly generated virions released into the culture supernatants were determined by quantification of HIV reverse transcriptase activities at different time points post-infection using the PCR-enhanced assay described recently (Pizzato et al., 2009). Only those mutant virions which had demonstrated spreading infection in H9 T-cells were analysed (since only these are likely to exhibit infection in the more stringent PBMC assay). As shown in Fig. 4, all of the examined mutant virions exhibited infection of PBMCs with kinetics which in all cases were similar to pNL-Wt.

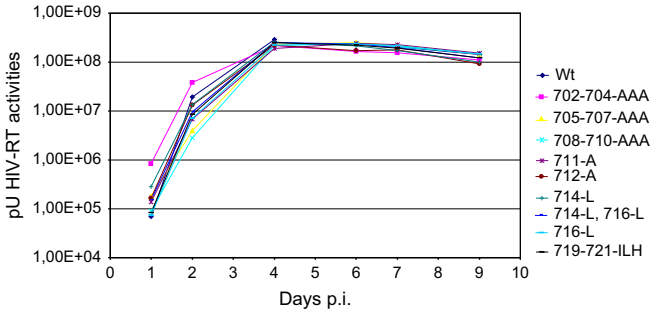


**Fig. 4.** Infection kinetics of pNL-Wt and mutant virions in activated human PBMCs. HIV reverse transcriptase activities (reflecting the amounts of released virions) in the media of infected activated PBMCs collected at different time points p.i. was quantitated as described in the methods section. Picomole units of HIV-RT are given as a function of time (in days). The identities of the virions analysed are given on the right.

## Discussion

Our chief focus in this study has been on the possible importance of the 13 membrane-proximal residues of the Env-CT (Env aa 706–718) for Env function and virus replication. Our initial mutational analysis (in total of aa 702–721), in which groups of 3 (in one case 2) aa were mutated to alanine, already indicated that the exact sequence of aa 702–710 and of 719–721 did not affect Env synthesis or function and did not inhibit mutant viral replication in activated primary blood lymphocytes. On the other hand, the chosen mutations between aa 711 and 718, although they did not inhibit Env synthesis or function, as measured by the abilities to mediate cell to cell fusion, did abolish viral replication in a T-cell line.

In the case of pNL-Env-711–713-AAA, this did not appear to be due to a negative effect on splicing since Gag and Env proteins were generated in the same amounts as Wt. However, as to expected from their drastically reduced splicing scores, in the cases of both pNL-Env-714–716-AAA and pNL-Env-717–718-AA, splicing at SA7 is defective and spliced transcripts in transfected cells differed dramatically from those seen in pNL-Wt (Fig. 2A). The defective Gag phenotype could be completely complemented when Rev protein was provided *in trans* by coexpression from a subviral vector.

However, although clearly defective in SA7 splicing and Rev production, both mutant proviruses still led to expression of functional Env proteins in a *rev*-independent manner. In fact mutation of *rev*, which abrogated Gag and Env expression in the Wt context, did not reduce Env expression in the context of pNL-Env-714–716-AAA. These unexpected observations, although actually not within the original focus of this study, demonstrate that the mode of Env expression in mutants pNL-Env-714–716-AAA and pNL-Env-717–718-AA is fundamentally different from that of Wt. Previously it has been demonstrated that a *cis*-acting motif, capable of repressing Env protein expression in a subgenomic context, is present within a 254 nt long sequence (restriction fragment HindIII-AvaI, nucleotide 8131–8383) mostly upstream of SA7 (nucleotide 8369) (Emerman et al., 1989). It is thus possible that the nucleotide point mutations in pNL-Env-714–716-AAA and pNL-Env-717–718-AA destroy this putative motif. If so, our data would narrow it down to a sequence of only 15 nucleotides (nucleotides 8354–8368), which functionally overlap with the polypyrimidine tract of SA7. Recently U2AF65 protein has been identified as one of the nuclear retention factors which bind to intron-containing pre-mRNAs (Takemura et al., 2011). It is thus plausible that the mutations within nucleotides 8354–8368 abolish this binding thus alleviating the requirement for Rev to counteract U2AF65-mediated retention of mutated 4 kb *env*-mRNAs. Intron-containing 4 kb *env*-mRNAs are 5′-terminally spliced and are thus loaded with at least one exon-junction complex which licenses such pre-mRNAs for nuclear export (Le Hir et al., 2000; Luo and Reed, 1999). An exon-junction complex, however, is lacking on the unspliced genomic viral RNA which is thus likely to be retained in the nucleus in the absence of Rev. These postulates would explain why Gag expression from pNL-Env-714–716-AAA and pNL-Env-717–718-AA is abrogated whereas Env expression still occurs. Future experiments will aim to clarify these issues.

In order to further analyse the importance of 711–718 for Env function and viral infectivity, we aimed to generate further mutations, which did not effect SA7 splicing. This was difficult for F717 and the chosen change, F717S, resulted in a reduced splicing score, a reduction in Gag expression to about 30% and rendered the mutant virus non-infectious. It was impossible to change Q718 without affecting splicing. It has thus not been possible in this study to evaluate the importance of the conservation of these two aa residues for Env protein function. However, the 8 double and single additional mutations within aa 711–716 all resulted in expression of normal amounts of Gag and functional Env. In fact mutant virions with changes in 4 of these positions (pNL-Env-711-A, pNL-Env-712-A, pNL-Env-714-L and pNL-Env-716-L) replicated in a T-cell line and showed replication kinetics similar to Wt in activated PBMCs. On the other hand, all three mutant virions encoding the Env substitution S713A (namely pNL-Env-711–713-AAA, pNL-Env-711-A, 713-A and pNL-Env-713-A) as well as the mutant virion encoding L715S exhibited reduced infectivities in Tzm-bl reporter cells and lack of replicative spread in H9 T-cells.

All of the mutant virions encoding S713A exhibited a reduction in Env incorporation into particles and this is presumably, at least in part, the reason for their replication defects. The processes leading to Wt HIV-Env incorporation into particles are still not completely understood. However, most models propose a role of the Env-CT domain either by direct or indirect interaction with Gag or by cotargeting of Env to Gag assembly sites (see Checkley et al. (2011) for review). A region spanning LLP2 has been implicated in directly mediating Env-CT binding to Gag (MA) (Murakami and Freed, 2000a) but many mutations and deletions outwith this region also reduce or abrogate Env incorporation into particles (e.g., (Piller et al., 2000)). The reasons for these latter incorporation defects are generally not known and this is also true here in the case of pNL-Env-713-A. Possibly minimal aberrant changes in mutant Env conformations may lead to the observed reduced Env incorporation and replication phenotypes.

**Table 2**
Summary of the properties of the generated mutants.

| Name | Score (1) | Amino acids 702–721 | | | | | | | | | | | | | | | | | | | | Env (2) | Gag (3) | Fusion (4) | Env in virion (5) | Tzm-bl (6) | H9-T-cells (7) | PBMC (8) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L 702 | S 703 | V 704 | V 705 | N 706 | R 707 | V 708 | R 709 | Q 710 | G 711 | Y 712 | S 713 | P 714 | L 715 | S 716 | F 717 | Q 718 | T 719 | H 720 | L 721 | | | | | | | |
| Wt | 7.15 | L | S | V | V | N | R | V | R | Q | G | Y | S | P | L | S | F | Q | T | H | L | + | + | + | + | + | + | + |
| 702–704-AAA | 7.15 | A | A | A | | | | | | | | | | | | | | | | | | + | + | + | + | + | + | + |
| 705–707-AAA | 7.15 | | | | A | A | A | | | | | | | | | | | | | | | + | + | + | + | + | + | + |
| 708–710-AAA | 7.15 | | | | | | | A | A | A | | | | | | | | | | | | + | + | + | + | +/- | + | + |
| 711–713-AAA | 7.10 | | | | | | | | | | A | A | A | | | | | | | | | + | + | + | +/− | +/− | − | n.d. |
| 711-A | 7.15 | | | | | | | | | | A | | | | | | | | | | | + | + | + | + | + | + | + |
| 711-A, 713-A | 6.66 | | | | | | | | | | A | | A | | | | | | | | | + | + | + | +/− | +/− | − | n.d. |
| 712-A | 7.72 | | | | | | | | | | | A | | | | | | | | | | + | + | + | + | + | + | + |
| 713-A | 6.66 | | | | | | | | | | | | A | | | | | | | | | + | + | + | +/− | +/− | − | n.d. |
| 714–716-AAA | −8.43 | | | | | | | | | | | | | A | A | A | | | | | | + | − | + | n.d. | − | − | n.d. |
| 714-L | 7.19 | | | | | | | | | | | | | L | | | | | | | | + | + | + | + | + | + | + |
| 714-L, 716-L | 7.05 | | | | | | | | | | | | | L | | L | | | | | | + | + | + | + | + | + | + |
| 715-S | 6.51 | | | | | | | | | | | | | | S | | | | | | | + | + | + | + | +/− | − | n.d. |
| 716-L | 7.01 | | | | | | | | | | | | | | | L | | | | | | + | + | + | + | + | + | + |
| 717–718-AA | −14.52 | | | | | | | | | | | | | | | | A | A | | | | + | − | + | n.d. | − | − | n.d. |
| 717-S | 5.58 | | | | | | | | | | | | | | | | S | | | | | + | +/− | + | n.d. | − | − | n.d. |
| 719–721-ILH | 8.43 | | | | | | | | | | | | | | | | | | I | L | H | + | + | + | + | + | + | + |

(1) SA7 splicing score as calculated using the MatEntScan tool (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html).(2) Env expression, (3) Gag expression, (4) Cell–cell fusion activity, (5) Env (gp120) incorporation into virions produced in H9 T-cells, (6) Infectivity in Tzm-bl reporter cells.(7) Infectivity in H9 T-cells, (8) Infectivity in activated PBMCs+ indicates values/activities as wild type, +/− indicates reduced in comparison to wild type, - indicates abrogated infectivity, "n.d." indicates "not determined".

The other non-replicative mutant, pNL-Env-715-S exhibited wild type phenotypes with respect to Env-mediated cell–cell fusion and Env incorporation but did not achieve replicative spread in H9 T-cells. L715 is the critical large hydrophobic residue in the membrane proximal YxxL endocytosis motif so that an obvious explanation is that an effect on endocytosis could be the reason for the replication defect of pNL-Env-715-S. On the other hand, the fact that in this study mutant pNL-Env-712-A, in which the essential tyrosine residue of the YxxL motif has been mutated, was still replication-competent speaks against this hypothesis. In numerous previous studies, the YxxL motif has been the focus of extensive analyses and viruses, mutated at Y712, analysed both with respect to Env localisation and virus infectivity. Most studies support the view that, in the context of the full-length Env-CT, destruction of the YxxL motif *alone* does not markedly alter Env subcellular localisation but that Y712 mutations in combination with mutation of the C-terminal 855, 856LL motif strongly increases Env surface expression (e.g., (Byland et al., 2007)). Concerning the replicative properties of Y712 mutants, depending on the amino acid change, the Env sequence context and the cell lines examined, differing results have been obtained. Thus, in some studies mutation of Y712 led to impaired replicative spread while in other studies, the respective mutant viruses replicated as well as wild type (Bhakta et al., 2011; Day et al., 2006; Lambele et al., 2007; West et al., 2002). At any rate, since in this study a functional YxxL motif is not a prerequisite for viral replication, the reason for the observed replicative defect of pNL-Env-715-S remains unknown.

It is perhaps surprising that mutation of the majority of the conserved membrane proximal Env-CT residues did not lead to defective phenotypes even in primary PBMCs. Our focus had been on aa 706–718 and, of these, aa 706–712, 714 and 716, although highly conserved within all HIV-1 strains (Fig. 1A), could be changed without abrogation of replicative properties. These results demonstrate that *in vivo* in an infected immunocompetent organism, critical viral features come to bear, which are dispensable even in primary cells in vitro. These are very likely to include targeting phenomena mediated by the membrane-proximal YxxL motif. However, since the further residues 706–711, 714 and 715, which have not been implicated in Env targeting, are also highly conserved, the roles which they may play remains a subject of speculation. For example, it is feasible that in some target cells *in vivo*, e.g., resting T-cells, macrophages etc., Env functions may be modulated by specifically expressed cellular proteins, which necessitate amino acid conservation in this membrane-proximal Env-CT region. On the other hand, it is possible that the conservation is, at least partially, driven by the necessity to maintain optimal nucleotide sequence conservation crucial to maintain secondary structures and signals required for regulatory fine tuning *in vivo*. These may be protein binding sites for additional regulatory proteins acting in concert with U2AF65 and/or UAP56 involved in RNA splicing and/or nuclear export (Takemura et al., 2011). On the other hand, as has become apparent recently (Watts et al., 2009), RNA architecture and secondary structure is also more globally important in governing native protein folding during translation. Again it is possible that this fine tuning is more impinged on *in vivo*. Experiments in the future may eventually throw some light on these issues.

## Materials and methods

### *Mutational analysis*

Mutations within the membrane proximal region of the Env-CT were generated in the backbone of the proviral construct pNL4–$3^{BH10env}$ (Wilk et al., 1992), referred to here as pNL-Wt, by standard PCR procedures and confirmed by sequencing. Nucleotide numbering given here is from pNL4-3 (Adachi et al., 1986) and Env amino acid (aa) numbering from strain BH10 Env (Ratner et al., 1985). The aa mutated and the sequences of the mutagenising oligonucleotides employed are given in Table 3. The nucleotide changes in mutant pNL-Env-719–721-ILH do not affect the sequence of the overlapping *rev* ORF. The oligonucleotide sequence introducing a stop codon in the *rev* gene and thus expression of a truncated Rev protein (Rev-Tr62) is also given in Table 3. It also introduces a *Pvu*II restriction site and three conservative aa changes within the overlapping Env protein. The respective proviral constructs with these mutations are designated pNL-rev$^{--}$ and pNL-Env-714–716-AAA-rev$^{--}$. The subviral construct, referred to here as pLTR-ctat/crev, which expresses Tat and Rev proteins from cDNAs has been described previously (Schaal et al., 1993). pMD.G is an expression vector for the G glycoprotein of vesicular stomatitis virus (VSV-G) (Naldini et al., 1996)

**Table 3**
Mutagenising oligonucleotides.

| Env mutants | Sequence |
|---|---|
| 702–704-AAA | GCTGTAGCTGCTGCAGTGAATAG |
| 705–707-AAA | CTTTCTGTAGCGGCTGCAGTTAGGCAGG |
| 708–710-AAA | GAATAGAGCTGCGGCGGGATAT |
| 711–713-AAA | GCAGGCAGCTGCACCATTATC |
| 711-A | GGCAGGCATATTCACCATTATC |
| 711-A, 713-A | GGCAGGCATATGCACCATTATC |
| 712-A | GGCAGGGAGCTTCACCATTATC |
| 713-A | GGCAGGGATATGCACCATTATC |
| 714–716-AAA | ATTCAGCAGCAGCGTTTCAGAC |
| 714-L | GATATTCACTATTATCGTTTCAG |
| 714-L, 716-L | GATATTCACTATTATTGTTTCAG |
| 715-S | GATATTCACCATCATCGTTTCAG |
| 716-L | GATATTCACCATTATTGTTTCAG |
| 717–718-AA | CCATTATCGGCTGCGACCCACCTCCC |
| 717-S | CATTATCGTCTCAGACCCACCTC |
| 719–721-ILH | GTTTCAGATTCCTCCACCCAA |
| Rev mutant | |
| Rev-Tr62 (Env L753S, L755V, I756S) | CGGATCCTCAGCTGTTAGCTGGGACG |

The names of the respective mutants and the sequence of the employed mutagenising oligonucleotides are given. The mismatched nucleotides are underlined.

### *Cells*

HEK 293 T cells and Tzm-bl indicator cells were cultivated in Dulbecco's modified Eagle's medium, 10% fetal calf serum (FCS) and H9 T-cells in RPMI-1640 medium (Gibco), 10% FCS. Peripheral blood mononuclear cells (PBMCs) from healthy donors were purified by Ficoll-Hypaque gradient centrifugation (Keppler et al., 2001). Several donors were pooled and activated at a density of $2\times10^6$ cells per ml in RPMI-1640 medium supplemented with 10% FCS, glutamine (2 mM) and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) together with 5 μg/ml PHA-P (Sigma) and 100 U IL-2 (Biomol) for three days as previously described (Baldauf et al., 2012). Afterwards, PMBCs were maintained in medium lacking PHA-P.

### *Analysis of viral proteins in cells and virions, cell–cell fusion activities*

HEK 293 T cells were transfected with proviral constructs and, at 48 h post-transfection (p.t.), cell lysates were prepared in gel electrophoresis sample buffer (containing 2% SDS, 5% mercaptoethanol) and aliquots of culture supernatants were stored at −70 °C for further use. Preparation of virus particles from infected H9 T-cells was performed as previously described (Emerson et al.,

2010; Holtkotte et al., 2007). Briefly, VSV-G pseudotyped Wt and mutant virions were generated in HEK 293 T cells by cotransfection of the respective proviral constructs with pMD.G. Aliquots of culture supernatants, collected at 48 h p.t., were used to infect H9 T-cells. After removal of input virions and washing (at 5 h p. i.), newly generated virion particles in the culture supernatants were collected at 48 h p.i., concentrated by ultracentrifugation through a cushion of 20% sucrose and lysed. Virion content in cell culture supernatants and in concentrated virion preparations were quantified by HIV p24-ELISA (Innogenetics, Belgium). Aliquots of cell and virion lysates were subjected to Western blot analysis employing rabbit anti-gp120 serum, mouse anti-gp41 (Chessie 8) or anti-p24 (183-H12-5C) as previously described (Emerson et al., 2010).

The abilities of the respective mutant Env proteins to mediate membrane fusion between cells was evaluated in principle as described previously (Holtkotte et al., 2006). Briefly, transfected HEK 293 T cells were cultivated with an approximately equal number of Tzm-bl indicator cells and 3 h later, luciferase activities (resulting from activation of the integrated HIV-LTR-luciferase cassette in Tzm-bl cells) measured (Bosch et al., 2009). The background on cocultivation of untransfected cells with Tzm-bl cells was negligible.

*Infections*

Virion infection levels on indicator Tzm-bl cells were evaluated as described previously (Bosch et al., 2009). Briefly, wells of Tzm-bl cells were infected in triplicate with doubling dilutions of virion-containing supernatants, in the presence of HIV protease inhibitor to prevent viral spread, and 48 h later, the cultures were fixed and stained for ß-galactosidase activity (from the integrated HIV-LTR-ß-galactosidase cassette in Tzm-bl cells). The number of stained cells, reflecting the number of infectious virions, were counted and titres calculated. The abilities of the mutant virions to mediate spreading infection in H9 T-cells were estimated as follows. Equal amounts of cell-free virions in the supernatants of transfected HEK 293 T cells (as assessed by CA-ELISA) representing very limiting amounts of infectious virus (MOI of pNL-Wt in H9 T-cells approx. 0.005) were employed to initiate infections. Infected cells were fixed on days 1, 2, 4, 6 and 7 p.i., stained with human anti-HIV serum (patient serum) and the number of infected cells estimated microscopically. Spreading infection was considered to have occurred when 100% of the H9 T-cells were stained positive on day 7.

$2\times10^5$ activated PBMCs in 200 μl medium containing IL-2 were infected with aliquots of supernatants of transfected HEK 293 T cells (equalized by p24-ELISA), representing, in the case of pNL-Wt, an MOI of 0.1 as measured by an infectivity assay on Tzm-bl cells. Infected PBMCs were washed 19 h p.i. with an excess of medium and taken up in 250 μl fresh medium containing IL-2. Aliquots (50 μl) of the supernatants were collected immediately (day 1) and stored at −−70 °C. The cultures were fed with a further 50 μl fresh medium containing IL-2 and cultured for a further 8 days. On days 2, 5, 6, 7 and 9, further 50 μl aliquots were collected and the cultures refed with 50 μl fresh medium containing IL-2. Quantification of released virions was achieved by analysis of HIV reverse transcriptase activities in the media using a one-step PCR-based assay as described (Pizzato et al., 2009). Briefly, supernatants and controls were lysed in 2× lysis buffer, followed by further dilutions in 1× dilution buffer prior to PCR analysis. A SYBR-Green I (Invitrogen)-based master mix including MS2 RNA (Roche) and MS2-specific primers (forward: 5′ TCCTGCTCAACTTCCTGTCGAG 3′; reverse: 5′CACAGGTCAAACCTCC-TAGGAATG 3′) were used to quantify HIV-1 reverse transcriptase activity in the supernatants with the following PCR parameters: 42 °C for 20 min, 95 °C for 2 min, 40 cycles at 95° for 5 s, 60 °C for 5 s, 72 °C for 15 s and 80 °C for 7 s (acquisition step) followed by melting curve analysis. Data acquisition and analysis were performed using Biorad CFX96 and Biorad CFX Manager software.

*Semi-quantitative RT-PCR analysis of viral mRNAs*

Total-RNA was isolated from HEK 293 T cells transiently transfected with the respective proviral constructs at 24 h p. t. by acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987). For reverse transcription 5 μg of total RNA were digested with 10 U of DNase I (Roche). DNase was subsequently heat-inactivated and cDNA synthesis performed using 200 U Superscript III RNAse H–Reverse Transcriptase (Invitrogen), 7.5 pmol oligo(dT)12–18 (Invitrogen), 20 U of RNAsin (Promega) and 10 mM of each deoxynucleoside triphosphate (Qiagen). The cDNA was subsequently subjected to PCR analysis using primer pairs 5′ CTT GAA AGC GAA AGT AAA GC 3′ (#1544, nucleotides 653–672) and 5′ CGT CCC AGA TAA GTG CTA AGG 3′ (#3392, nucleotides 8469–8489) for the amplification of intronless 2 kb mRNA species and #1544 and 5′ CAA TAC TAC TTC TTG TGG GTT GG 3′ (#640, nucleotides 6456–6478) for intron-containing 4 kb mRNA species, respectively. RT-PCR products were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide.

## Acknowledgments

We thank Martin Löchelt, Deutsches Krebsforschungszentrum for fruitful discussion. Monoclonal antibodies against gp41 (Chessie 8) and against p24 (H183–H12–5C) were obtained from the NIH AIDS Research and Reference Reagent Program. These studies were supported in part by the DFG (SCHA 909/3-1), the Heinz Ansmann Foundation for AIDS research, Düsseldorf (H.S.), and Jürgen Manchot Stiftung (M.W., H.S.).

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