The Human Immunodeficiency Virus (HIV) *gag* Gene Product p18 Is Responsible for Enhanced Fusogenicity and Host Range Tropism of the Highly Cytopathic HIV-1-NDK Strain

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Formation of large syncytia and rapid cell killing are characteristics of the Zairian human immunodeficiency virus type 1 isolate HIV-1-NDK, which is highly cytopathic for CD4$^+$ lymphocytes in comparison with the HIV-1-LAV prototype. Chimeric viruses containing different combinations of HIV-1-NDK genetic determinants corresponding to the splice donor, the packaging signal, and the coding sequence of the p18^{gag} protein together with the HIV-1-NDK $EcoRI_{5278}$-$XhoI_{8401}$ fragment were obtained by polymerase chain reaction-directed recombination. Phenotypic analysis of recombinant viruses indicated that 75 amino acids from the N-terminal part of HIV-1-NDK p18^{gag} protein together with the HIV-1-NDK envelope glycoprotein are responsible for enhanced fusogenicity of HIV-1-NDK in CD4$^+$ lymphocytes as well as for enhanced infectivity of HIV-1-NDK in some CD4$^-$ cell lines. The HIV-1-NDK splice donor/packaging sequence and the sequence encoding the *gag* protein p25 were not important for the variation observed in HIV-1 fusogenicity.

Human immunodeficiency virus type 1 (HIV-1) isolates vary widely in their cytopathogenicity (4, 11, 14, 17, 25, 39) and in their host range in CD4-positive and -negative cells (10, 15, 26, 34). Elucidation of the genetic control of these phenotypic properties is of fundamental importance for our understanding of HIV-1 virulence and attenuation as well as for appropriate selection of virus strains for possible HIV-1 vaccines.

Numerous genetic studies have clearly demonstrated the importance of the *env* gene for induction of HIV-1 cytotoxicity and host range tropism (8, 21–23, 26, 34, 35, 38). In addition to the *env* gene, two control mechanisms of HIV-1 gene expression are reported to influence HIV-1 cytopathogenicity; they include (i) a possible negative regulatory activity of the *nef* gene product (3, 24) and (ii) differences in efficiency of HIV transactivation systems involving long terminal repeat, *trans*-acting response element, and *rre* sequences and *tat* and *rev* gene products (4, 9). More recent studies, however, indicate that differences among activities of *nef* genes (8, 12, 20) are not associated with variations in the cytopathogenicity of HIV-1 isolates.

Formation of large syncytia, rapid cell killing, and early onset of replication are characteristics of the Zairian virus strain HIV-1-NDK, an isolate highly cytopathic for CD4$^+$ lymphocytes (14, 17, 18, 36, 37) in comparison with the HIV-1-BRU prototype. By analyzing the properties of recombinant chimeric viruses derived from HIV-1-BRU and HIV-1-NDK, we have recently shown (17, 36) that the simultaneous expression of the HIV-1-NDK $BssHII_{255}$-$SpeI_{1042}$ and $EcoRI_{5278}$-$XhoI_{8401}$ fragments is important for development of the enhanced fusogenicity of HIV-1-NDK. Implication of the $EcoRI_{5278}$-$XhoI_{8401}$ fragment, which contains the HIV-1 *env* gene, was not surprising. However, the importance of genetic determinants present in the $BssHII_{255}$-

$SpeI_{1042}$ fragment was not anticipated. As far as we know, this is the first demonstration of the direct involvement of an HIV-1 structural gene other than *env* in the induction of cytopathic changes by HIV-1 (17, 36).

The genetic structure of the HIV-1-NDK $BssHII_{255}$-$SpeI_{1042}$ fragment is actually quite complex. It contains determinants of a splicing donor (nucleotide [nt] 287), an RNA packaging signal (nt 288 to 318), and sequences coding for protein p18 (nt 334 to 726) and the N-terminal part of the *gag* protein p25 (nt 727 to 1042). A small specific deletion of 3 amino acids (aa) not observed in other sequenced HIV-1 isolates was detected within the C-terminal portion of the HIV-1-NDK protein p18 (37). Any of these regions could conceivably be involved in induction of HIV1 cytopathogenicity. In this study, we have attempted to localize the minimal portions of the HIV-1-NDK $BssHII_{255}$-$SpeI_{1042}$ fragment responsible for its enhanced fusogenic potential in a cell culture. We have also attempted to determine whether enhanced fusogenicity of HIV-1-NDK, related to the presence of specific $BssHII_{225}$-$SpeI_{1042}$ sequences, is important for the mechanism of HIV-1 cell entry and host cell tropism, especially in CD4-negative cell lines (7). The latter question is especially relevant to our recent finding that HIV-1-NDK is able to infect CD4$^+$ lymphocytes in the presence of anti-CD4 antibodies (29). Phenotypic analysis of recombinant viruses indicated that 75 aa from the N-terminal part of the HIV-1-NDK p18^{gag} protein together with the HIV-1-NDK envelope glycoprotein are responsible for enhanced fusogenicity of HIV-1-NDK in CD4$^+$ lymphocytes as well as for enhanced infectivity of HIV-1-NDK in some CD4$^-$ cell lines.

The construction of parental HIV proviruses pNL4-3 (2), pLB (HIV-1-LAV) (18), and pY-NDK (HIV-1-NDK) (18), their transfection into cells, and preparation of the virus were recently described (18). These cloned viruses as well as the HIV-1-BRU prototype isolate (5) and the highly cytopathic HIV-1-NDK isolate (14) were propagated in the permissive CEM continuous cell line. The cell line MT4,

which is nonproductively infected with human T-cell lymphotropic virus type I, as well as cell lines H9 and COS, was cultivated as described earlier (18, 28).

The RD human rhabdomyosarcoma cell line was obtained from A. Srinivasan (Wistar Institute, Philadelphia, Pa.); a keratinocyte cell line, HACAT, derived from human skin obtained during melanoma surgery, was obtained from H. Fussenick (German Cancer Research Institute, Heidelberg, Germany). All cells were maintained in Dulbecco's modified Eagle's medium with 10% heat inactivated fetal calf serum, L-glutamine, and antibiotics and infected as described earlier (7). The lack of CD4 expression in these cells was assessed by cell surface immunofluorescence and Northern (RNA) blotting (7).

Recombinant provirus molecules derived from cloned HIV-1-LAV and HIV-1-NDK DNA (18) were constructed by reciprocal exchange of genetic material using conserved $BssHII_{255}$, $SpeI_{1042}$, $EcoRI_{5278}$, and $XhoI_{8401}$ sites (Fig. 1). For the regions lacking suitable restriction sites but exhibiting a nucleotide homology, a polymerase chain reaction (PCR) was used to create the desired recombinant fragments. In the first step, two adjacent parts of the genome derived from different parental molecules, limited on their outer ends by the conservative restriction sites, and containing an overlapping sequence of 15 to 20 nt at their proximal ends were separately amplified. The products of the first reaction were mixed, and the recombinant fragment was generated in the second step by using oligonucleotide primers covering the restriction sites used for the insertion into the infectious provirus molecule. The amplification primers mapped to the following positions of the HIV-1-LAV (41) and HIV-1-NDK (37) genomes: the DNA plus-strand primers were LAV1 (nt 731 to 750), LAV3 (nt 246 to 266), LAV6 (nt 577 to 597), NDK1 (nt 247 to 267), NDK3 (nt 337 to 357), and NDK4 (nt 680 to 700); the DNA minus-strand primers were LAV2 (nt 1059 to 1040), LAV4 (nt 356 to 336), LAV5 (nt 697 to 677), NDK2 (nt 732 to 717), and NDK5 (nt 595 to 575). Note the different numbering of homologous sequences of HIV-1-LAV and HIV-1-NDK caused by HIV-1 genomic variation. The primers LAV3 and NDK1 contained a $BssHII$ restriction site, and LAV2 contained an $SpeI$ restriction site. The resulting fragments were digested with $BssHII$ and $SpeI$ to generate cohesive ends and inserted by homologous recombination into the provirus pNLDE (36), which contains the HIV-1-NDK-derived $EcoRI_{5278}$-$XhoI_{8401}$ fragment inserted into the pNL4-3 (2) sequence. Recombinant provirus clones were screened by restriction enzymes, and their $BssHII$-$SpeI$ fragments generated by PCR were sequenced. Chimeric viruses were recovered after transfection of COS cells by provirus molecules and cocultivation with CEM cells as described in detail previously (18).

Virus concentrations in cell supernatants were determined by reverse transcriptase (RT) assay (5, 28) and by the p25^{gag} antigen capture technique (29). Virus titers were determined by end point dilution assay as measured by RT and occurrence of cytopathic effects. The fusogenicities of different recombinant viruses were determined by syncytium formation on MT4 or H9 cells, both of which are highly sensitive to HIV infection (17, 18, 28).

Cell monolayers at about 80% confluency in 25-cm² flasks were infected with 0.5 ml of filtered (Millipore; 0.45-μm pore size) virus at 37°C for 4 h, washed, and incubated further in Dulbecco's modified Eagle's medium. The infected cells were passaged twice by trypsinization, always after a 5-day period. After the second trypsinization, the cells were seeded in 24-well tissue culture plates. Two days later, 5 ×
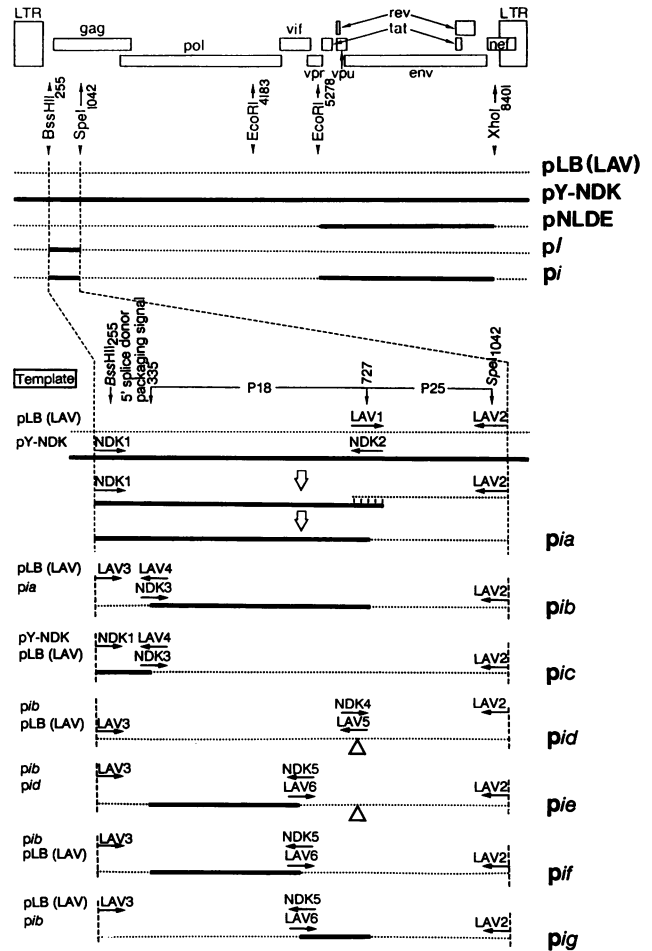

FIG. 1. PCR-directed recombinations in the HIV-1 provirus $BssHII_{255}$-$SpeI_{1042}$ fragment. Recombinant provirus molecules derived from cloned pLB (HIV-1-LAV prototype) (. . .) and pY-NDK (highly cytopathic HIV-1-NDK) (━━━) were constructed by using the conserved $BssHII_{255}$, $SpeI_{1042}$, $EcoRI_{5278}$, and $XhoI_{8401}$ sites (18, 36). The positions of these restriction sites are indicated for the HIV-1-NDK sequence. The schematic locations and orientations of the oligonucleotide primers used for amplification of original and derived templates in the primary PCR are indicated by arrows. Only external primers were used during recombination of these primary products in a secondary PCR. Parental templates are indicated and aligned with the respective oligonucleotides. The recombinant products of the secondary PCR were treated with $BssHII$ and $SpeI$ and inserted into pNLDE to generate chimeric proviruses. The open triangles under proviruses pid and pie indicate the position of the TGH deletion introduced into the HIV-1-LAV sequence. LTR, long terminal repeat.

10⁵ indicator CEM cells were added to each well, and cocultivation was continued in RPMI 1640 medium. The cell supernatants were assayed for RT activity before each passage.

Chimeric viruses containing combinations of HIV-1-NDK sequences corresponding to splice donor and packaging signals, p18^{gag} protein, and the HIV-1-NDK $EcoRI_{5278}$-$XhoI_{8401}$ fragment were obtained by PCR-directed recombination (Fig. 1). The strategy of construction of recombinant proviruses is illustrated for recombinant provirus pia (Fig. 1), which contains HIV-1-NDK-derived sequences from the splice donor up to the C terminus of protein p18 followed by

TABLE 1. Replication properties of recombinant viruses

Virus recombinant	CPE induction in MT4 cells[a]		Virus production in CEM cells[b]		
	Morphology of syncytia	Days on which CPE first observed (1 TCIU)[c]	Day on which RT production peaked		Intensity
			1 TCIU	10 TCIU	
LB(LAV) (m)	+	10	16	14	H
Y-NDK (a)	+++	6	10	10	M
NLDE (k)	+	10	16	14	L
i	+++	9	14	10	L
ia	+++	9	14	10	L
ib	+++	9	14	14	L
ic	+	10	14	10	L
id	+	10	14	10	L
ie	+++	9	ND		ND
if	+++	9	ND		ND
ig	+	9	ND		ND

[a] Induction of syncytia by recombinant viruses in MT4 assay. A total of 10^5 MT4 cells was infected with recombinant viruses. The number indicates the day on which the cytopathic changes were first observed at the virus limit dilution. The sizes of syncytia were estimated each day for 2 weeks. The morphology of syncytia in MT4 cells is indicated by + for standard-size syncytia induced by the HIV-1-LAV prototype and by +++ for large syncytia induced by the highly cytopathic strain HIV-1-NDK (17). CPE, cytopathic effect.

[b] A total of 10^5 CEM cells was infected with the indicated amount of recombinant virus. Virus recombinants produced a quantity of virus equivalent to $>10^5$ (low [L]), $>2.5 \times 10^6$ (medium [M]), and $>5 \times 10^6$ (high [H]) cpm of RT per ml. ND, not determined.

[c] TCIU, tissue culture infectious unit.

FIG. 2. Replication of recombinant viruses NLDE (□), ib (▲), and ic (△) and parental clones LB(LAV) (O) and Y-NDK (●) in CEM cells as measured by production of RT activity in cell supernatants. A total of 10^6 cells was infected with 1 tissue culture infectious unit of each virus.

the HIV-1-LAV-derived sequence encoding gag protein p25. Fragments of HIV-1-LAV and HIV-1-NDK were first separately amplified with the LAV1-LAV2 and NDK1-NDK2 oligonucleotide primer pairs and pLB(LAV) and pY-NDK (18) plasmid templates, respectively. Both PCR products were purified, mixed, and amplified by further taking advantage of an overlapping sequence of 13 nt and using external primers NDK1 and LAV2 in the absence of internal primers LAV1 and NDK2. The resulting fragments were inserted into the provirus pNLDE (36), which contains the HIV-1-NDK-derived $EcoRI_{5278}$-$XhoI_{8401}$ fragment (Fig. 1). In a similar way, proviruses pib, pic, pid, pie, pif, and pig, which contain recombinant sequences of protein p18, were obtained.

Chimeric viruses ia, ib, ic, id, ie, if, and ig were assayed for formation of syncytia in MT4 cells (Table 1). The results indicate that the N-terminal part of $p18^{gag}$ of HIV-1-NDK together with genetic determinants localized in the HIV-1-NDK-derived $EcoRI_{5278}$-$XhoI_{8401}$ fragment was required for enhanced fusogenicity of HIV-1-NDK. Recombinants ia and ib (Table 1), which encode HIV-1-NDK-derived $p18^{gag}$ together with the HIV-1-NDK $EcoRI_{5278}$-$XhoI_{8401}$ fragment, as well as recombinants ie and if (Table 1), which contain the first 75 aa derived from the N-terminal part of p18, induced the formation of large syncytia. The first 110 noncoding nucleotides of the $BssHII_{255}$-$SpeI_{1042}$ fragment, containing the HIV-1 5' splice donor and packaging signal, were not important for variation of HIV-1 fusogenicity. Recombinant ic (Table 1) contained the HIV-1-NDK splice donor and packaging sequences and formed prototype-like small syncytia. In contrast, recombinants ib, ie, and if (Table 1) contained the splice donor and packaging sequence derived from the pLB(LAV) prototype and formed large syncytia.
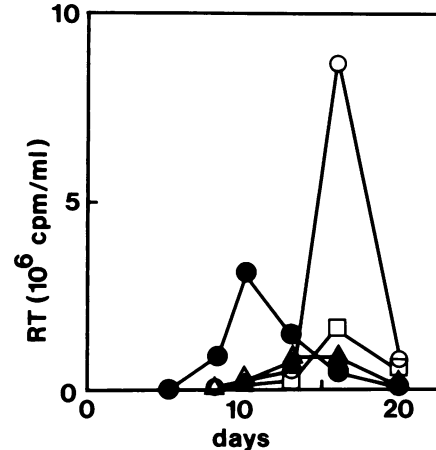
The last 57 aa residues of the C-terminal part of p18, including the deletion of 3 aa (TGH; aa 122 to 124) specific for HIV-1-NDK, were also not important for variation of HIV-1 fusogenicity. This 3-aa deletion introduced into the HIV-1-LAV sequence (provirus pid) or naturally present within the last 54 aa (aa 76 to 129) of the C-terminal part of HIV-1-NDK p18 (provirus pig) resulted in formation of prototype-like syncytia. Additional evidence indicates that the C-terminal part of p18 does not determine the fusogenic phenotype. A 7-aa repeat, AQQAAAD (aa 115 to 121) was inserted accidentally upstream of the TGH deletion in one subclone during the construction of provirus pid. This PCR artifact was capable of the formation of infectious virus with a prototype-like phenotype (data not shown).

Parental recombinant virus NLDE and recombinant i (Fig. 1) were recently characterized according to the time and the magnitude of RT production as "slow/low" viruses (17). Therefore, it was not surprising that all of the PCR-constructed recombinants, ia to ig (Fig. 2; Table 1), displayed similar replication properties. Originally we speculated that the replication rate in recombinant i could result from a superposition of an enhancing effect of the HIV-1-NDK RNA leader sequence containing the splice donor and packaging sequences and down regulation by the rest of the sequences of the $BssHII_{255}$-$SpeI_{1042}$ fragment. However, the results indicate that the replication rate did not correlate with the presence of any part of the $BssHII_{255}$-$SpeI_{1042}$ fragment derived from the HIV-1-NDK sequence.

Two CD4-negative human cell lines derived from a rhabdomyosarcoma (RD) and from human skin (HACAT) were infected with parental cloned HIV-1-LAV and HIV-1-NDK and with chimeric viruses NLDE and i (Fig. 3). None of the target cell lines produced virus in quantities sufficient to be detected directly by RT or p25 assays in cell-free supernatants (7). However, successful infection of each cell line with complete HIV-1-NDK as well as with recombinant i (Fig. 1) could easily be detected after cocultivation with HIV-sensitive indicator CEM cells. Replication of recombinant NLDE, which lacks HIV-1-NDK-derived protein p18, as well as of the prototype virus was not detected in either of the cell lines. These data provide qualitative and not quan-
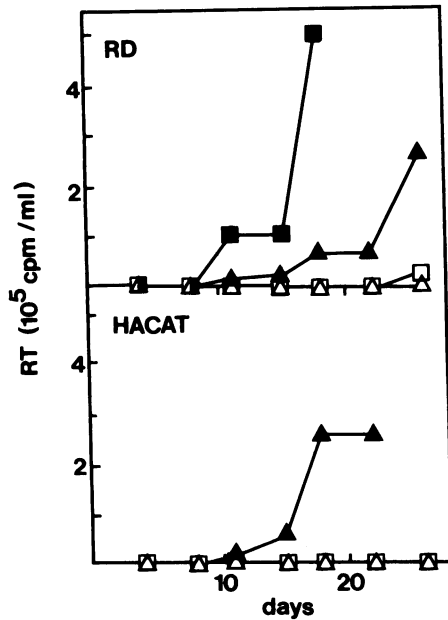
FIG. 3. Cocultivation of rhabdomyosarcoma (RD) and human keratinocyte (HACAT) cells infected with HIV-1-BRU (□), HIV-1-NDK (■), and recombinants NLDE (△) and *i* (▲) with CEM cells. Cocultivation started after two passages of infected cell lines (8 days after infection). HIV-1 production was detected by RT assay.

titative information; they demonstrate the production of HIV by the target cells as well as by the indicator cells. Moreover, the same virus strains, Y-NDK, LB(LAV), NLDE, and *i*, differed strongly in their growth properties in CEM cells (Table 1) (17). However, within this limitation, the results clearly indicate that p18^{gag} is involved in variation of the host range tropisms of at least some HIV strains. No cytopathic changes or significant changes in growth characteristics of RD or HACAT cells were observed up to 30 days after infection.

Our data have demonstrated the importance of the p18^{gag} gene in the variation of HIV-1-induced cytopathic effects and HIV-1 infectivity in CD4-negative cell lines. Protein p18, which forms an isometric scaffold for HIV-1 virions, is in contact with core protein p25 as well as with the transmembrane glycoprotein gp41. As in virions, p18^{gag} protein could modify the spatial conformation of envelope glycoproteins on cell surfaces during virus budding. Epitopes of p18^{gag} have recently been detected on the surfaces of intact infected cells (32). The resulting conformational changes in the HIV-1 envelope glycoprotein, especially in its gp41 subunit, could influence the fusogenicity of HIV-1 and consequently its host range tropism in CD4-negative cell lines. Direct interaction between the outermost protein of the *gag* complex and the retrovirus envelope glycoprotein on the surface of infected cells has been clearly demonstrated for murine leukemia virus (31). It is also possible that unknown structures or signals present in the RNA sequence corresponding to 75 aa from the N-terminal part of HIV-1-NDK p18^{gag} protein influence development of cytopathic effects. Studies of a direct mechanical interaction and *trans* complementation of the p18^{gag} protein with *env* products are necessary to clarify this problem.

Sequence analysis revealed differences between HIV-1-BRU and HIV-1-NDK in 21 aa residues of protein p18.

Among them, substitutions E→K (aa 12), R→T (aa 15), K→A (aa 30), and Q→E (aa 90), the last resulting in a change in amino acid charge, and deletion of 3 aa (TGH; aa 122 to 124) seem to be the most significant. In this study we have found that only 75 aa from the N-terminal part of p18 are responsible for the variability of cytopathic effects. Whereas substitution E→K was also found in other African isolates, the other two changes, R→T and K→A, were specific for HIV-1-NDK.

Two important structural elements have been localized to the N-terminal part of p18. (i) The targeting of the HIV *gag*-encoded precursor at the cell plasma membrane is dependent on the attachment of a myristic acid moiety to its N-terminal glycine residue (16). (ii) A short stretch of basic amino acids (aa 26 to 32), highly conserved in both HIV-1 and HIV-2 as well as in simian immunodeficiency virus, simian virus 40 large T antigen, polyomavirus VP 213, and the adenovirus 72,000-molecular-weight DNA-binding protein, was suggested to serve as a karyophilic signal for the transport of Pr55^{gag} to the nucleus (13). Transport of parental p18^{gag} protein into the cell nucleus shortly after virus internalization was recently described by Sharova and Bukrynskaya (33). Whereas the N-terminal glycine is conserved in both parental HIVs, the important amino acid substitution K→A (aa 30) is localized in the basic amino acid stretch of HIV-1-NDK only.

p18^{gag} contains epitopes which have the capacity to elicit a wide spectrum of host responses (1, 19, 27, 30, 40). Targets for cytotoxic lymphocytes (1, 40) have recently been localized to the C-terminal portion of p18^{gag}. In addition, sequence similarity to membrane-associated proteins of RNA viruses in this part of p18 has been described (6). The C-terminal part of p18^{gag}, including a specific deletion of 3 aa (TGH; aa 122 to 124) in HIV-1-NDK, was not important for variations in the cytopathic phenotype. Immunological tolerance caused by sequence homology between human thymosin alpha and p18^{gag} could explain the relatively low degree of sequence divergence in this part of the HIV genome, resulting in a stable cytopathic phenotype.

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