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Nucleotide sequence of HIV1-NDK: a highly cytopathic strain of the human immunodeficiency virus

(Recombinant DNA; genomic variability; Zaïrian isolates; AIDS; phage λ vector; syncytia)

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SUMMARY

A highly cytopathic strain of HIV1, named HIV1-NDK, has been isolated from a Zaïrian patient affected with AIDS. This isolate is 10^4 times more cytopathic and infectious than the prototype. To correlate the high cytopathic properties of this strain with genetic variations, we have cloned and sequenced the genome of this isolate. The principal feature which could be drawn from the fine analysis of the HIV1-NDK sequence is that the variability is not clustered in one particular region but rather spread out all along the genome. Only minor differences seem to be responsible for the acute biological effect of HIV1-NDK.

INTRODUCTION

The genomic structure of the AIDS virus, human immunodeficiency virus (HIV), has been well established. The nt sequences of LAV1 and HTLVIII B

prototypes have revealed a complex organization, including three structural genes and at least five regulatory genes (Wain-Hobson et al., 1985; Ratner et al., 1985). The same genomic organization, although retained in several variants from North

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Abbreviations: aa, amino acid(s); AIDS, acquired immunodeficiency syndrome; bp, base pair(s); CPE, cytopathic effect; CD4, cluster of differentiation N4; DMEM, Dulbecco's modified

minimal Eagle's medium; DMSO, dimethyl sulfoxide; EM, electron microscopy; Env, envelope glycoprotein gp120 of HIV; *env*, gene encoding Env; FCS, fetal calf serum; gp, glycoprotein; HIV, human immunodeficiency virus; HTLV, human T lymphotropic virus; kb, kilobase(s) or 1000 bp; LAV, lymphadenopathy-associated virus; LTR, long terminal repeat; nt, nucleotide(s); ORF, open reading frame; PBL, peripheral blood lymphocytes; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; PRO, HIV1 prototype; RT, reverse transcriptase.

America, Europe and Africa, has been nevertheless accompanied by a great deal of genetic heterogeneity, especially in the 5' part of the *env* gene encoding the external glycoprotein gp120 (Env) (Alizon et al., 1986; Starcich et al., 1986; Willey et al., 1986).

Besides their genomic variability, all isolates are characterized by their cytopathic effect (CPE) on CD4⁺ cells, consisting of a massive degeneration of cell population; several studies (e.g. Sodroski et al., 1986a; Kowalski et al., 1987) have indicated that CPE is associated with giant cells (syncytia-forming cells), resulting from fusion through CD4 and Env interaction. A loss or gain in the cytopathogenicity of HIV isolates has recently been demonstrated by several groups of investigators (Asjö et al., 1986; von Briesen et al., 1987), which tends to indicate that HIV1 biological variability could occur within a single patient. Cheng-Mayer et al. (1988) have described naturally-occurring variants of HIV1 isolates, obtained at intervals during infection in individuals: these variants exhibit an enhanced cytopathic effect in vitro, which correlates with the progression of the disease. The only biochemical change detected has been found in the M_r of the Env glycoprotein, probably due to variations in the glycosylation. On the other hand, reduced CPE has been demonstrated after introduction of deletions into the 3' part of the viral genome, spanning the *env* and the *nef* genes (Fischer et al., 1986). Mutants resulting from deletions in several parts of the *env* gene have implicated the critical importance of some aa for viral infectivity and/or cytopathogenicity, thus suggesting 'distortion' between viral replication and induction of syncytia (Kowalski et al., 1987). Other studies (Leonard et al., 1988) had demonstrated that CPE has not only been due to cell fusion, but might also result from a direct CPE of the virus during its release from infected cells. It is not clear, as yet, whether this latter mechanism of CPE could be attributed to a specific viral genetic function, as syncytia formation is attributed to the CD4-Env interaction.

Attempts to correlate the CPE with a relevant genetic variation of the viral genome led us to choose, from our panel of HIV1 strains, a particular HIV1 isolate which presents all characteristics of a highly cytopathogenic strain. This strain, HIV1-NDK, was isolated from a Zaïrian patient with AIDS (Ellrodt et al., 1984) who transmitted the virus to his wife, who in her turn transmitted it to her lover. All pro-

gressed quickly from seropositivity to AIDS and to death. Inoculation of this HIV1-NDK isolate into chimpanzee induced a T4 depletion (P. Fultz, personal communication). It should be noted that HIV1-NDK is the only strain able to develop such an in vivo effect, which represents an additional argument for our interest in it.

In this paper, we describe the complete nt sequence of the HIV1-NDK strain and the comparison of its aa sequence with that of an HIV1 prototype. This data leads us to suggest that only minor variations are responsible for its biological effect.

MATERIALS AND METHODS

(a) Virus isolation

HIV1-NDK was isolated from peripheral blood lymphocytes as previously described (Ellrodt et al., 1984). The virus was then propagated in the continuous permissive CEM cell line (Foley et al., 1965). Viral production was monitored by RT assay, immunofluorescence and electron microscopy (Barré-Sinoussi et al., 1983; Rey et al., 1984).

(b) Quantitation of CPE

The cytopathogenicity of the HIV viral strains was determined by superinfection of the HTLV1 positive cell line MT4 (Harada et al., 1985; Rey et al., 1987). The number of surviving cells was determined by trypan blue exclusion seven days after infection.

(c) Molecular cloning and genomic library

High M_r DNA extracted from HIV1-NDK-infected CEM cells was partially digested with *Hind*III in order to obtain fragments ranging from 10 to 15 kb in size, which were then purified through a 10 to 40% sucrose gradient (Maniatis et al., 1982). The product of the partial digestion was ligated into the arms of the *Hind*III-cleaved λ L47 phage. The resulting large molecules were packaged with Gigapack-Gold extract, according to the manufacturer's instructions (Stratagene, La Jolla, CA). The library was screened with the pBT1 probe corresponding to the large *Sac*I fragment of the HIV1 prototype (Alizon et al., 1984)

employing the plaque-filter annealing method (Benton and Davis, 1977). The plasmid pBT1 used for these experiments was kindly provided by Dr. S. Wain-Hobson. Positive phages were purified and amplified, and mapping was performed by the double-digestion method (Maniatis et al., 1982) and by Southern blot hybridization, with several sub-clones derived from pBT1 genome.

(d) Nucleotide sequencing

5 μ g of DNA corresponding to the viral parts of the recombinant HIV1-NDK phage was sonicated to obtain 500-bp fragments. After filling-in of the ends with the PolIk, the fragments were subcloned into the *Sma*I site of the M13mp18 vector (Messing and Vieira, 1982). Sequencing was performed according to the dideoxy chain termination method, using a universal primer (Sanger et al., 1977).

(e) Computer analysis

All computer programs were performed using the Pasteur MV 8000 computer. Shotgun programs were used in order to determine the overlaps of the sonicated fragments. Nucleic acid and protein alignment were carried out by NUCALN and PRTALN programs (Wilburg and Lipman, 1983).

TABLE I

Comparison of cytopathogenicity and infectivity of HIV1 prototype and HIV1-NDK

| Dilution of viral stock ^a | Presence of syncytia in MT4 cells ^b (7 days after infection) | | Detection of infection in PBL ^c (7 days after infection) | |
|--------------------------------------|---|----------|---|----------|
| | HIV1 PRO | HIV1-NDK | HIV1 PRO | HIV1-NDK |
| 0 | + | + | + | + |
| -1 | + | + | + | + |
| -2 | + | + | + | + |
| -3 | + | + | + | + |
| -4 | - | + | - | + |
| -5 | - | + | - | + |
| -6 | - | + | - | + |
| -7 | - | + | ND | ND |
| -8 | - | - | ND | ND |

^a Tenfold dilutions of both strains were performed in RPMI 1640 medium from a stock (cell-free supernatant) titrating at 3×10^6 cpm/ml and 10^6 cpm/ml of RT activity for HIV1 prototype and HIV1-NDK, respectively.

^b +, Presence of syncytia; -, absence of syncytia; PRO, prototype.

^c +, detection of viral production 7 days after infection; -, no viral production 7 days after infection; PRO, prototype; ND, not done.

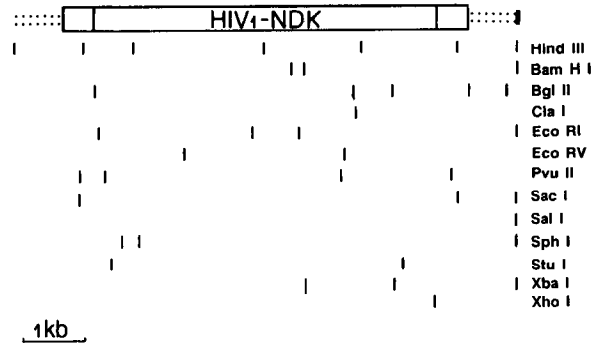


Fig. 1. Restriction map of plasmid pNDK containing the full length HIV1-NDK genome subcloned into the *Hind*III site of pUC18 (see RESULTS, section b). Dotted double lines indicate the cellular flanking regions, the vertical heavy bar represents (at the right terminus) the multiple cloning site. The two open squares at each extremity of the viral genome indicate the LTRs. Restriction mapping was performed, as described in MATERIALS AND METHODS, section c.

(f) Eukaryotic cell transfections

COS-1 cells (Gluzman et al., 1981) were seeded at a density of 4×10^6 cells per 75 cm² flask, 24 h prior to transfection. Transfection for subsequent DNA analyses was carried out by the calcium phosphate coprecipitation technique (Graham and Van der Eb, 1973), using 10 μ g of plasmid DNA. After 12 h the cell culture was shocked with 10% DMSO for 1 min, prior to recovery in DMEM with 10% FCS. CEM

cells were added to the COS-1 cells 16 h after transfection and cocultivated for 21 days, while testing RT activity in cell-free supernatant.

RESULTS AND DISCUSSION

(a) High cytopathogenicity of the HIV1-NDK strain

To compare the CPE of HIV1-BRU isolate (HIV1 prototype) and HIV1-NDK, serial dilutions of virus using cell-free supernatant were used to infect MT4 cells. The presence of syncytia in the cell culture was determined 7 days after infection. The results shown in Table I (left side) reveal that 10^{-7} diluted HIV1-NDK stocks were able to induce syncytia formation, while HIV1 prototype diluted below 10^{-3} did not lead to any CPE. To demonstrate that syncytia are due to viral replication, infection of PLBs was performed with serial dilutions of both strains. The results shown in Table I (right side) indicate that the same dilutions which induce syncytia formation on MT4, are able to infect PBL, suggesting that HIV1-NDK is 10^4 times more cytopathic and infectious than the prototype.

Level of RT activity and percentage of infected cells are the same in continuous cell lines infected with HIV1 prototype or with HIV1-NDK, suggesting that viral production is quantitatively similar for both

strains. Furthermore, EM studies did not show more budding on the surface of HIV1-NDK-infected cells (results not shown). These data suggest that the higher infectivity and cytotoxicity of the HIV1-NDK isolate could be attributed to qualitative properties of the virus and not to higher viral replication.

(b) Molecular mapping of the HIV1-NDK genome

Recombinant phage picked up from the λ L47 genomic library (see MATERIALS AND METHODS, section c, for details) were subcloned, using partial *Hind*III digestion in pUC18 plasmid. The restriction map of one of the recombinant plasmids, pNDK, is presented in Fig. 1. Taking into account that *Sac*I and *Hind*III sites have always been found in the LTRs of all HIV1 isolates as yet published, digestion of pNDK with these enzymes was performed. The *Sac*I digest leads to the identification of an internal viral fragment of 9.1 kb and the hybridization of *Hind*III fragments with the pBT1 probe (results not shown) clearly identified the junction fragments within the cellular genome. Restriction analysis indicates that the HIV1-NDK genome is different from other known HIV1 isolates.

In addition, the complete HIV1-NDK genome with two entire LTRs flanked by cellular genome was biologically active, since virus was recovered after co-culture of CEM with transfected COS cells (results not shown).

TABLE II

Quantification of amino acid sequence homology between homologous proteins of different isolates^a

| ORF | HIV1-NDK/HIV1 PRO ^b (%) | HIV1-NDK/HIV1-ELI ^c (%) |
|-----------|---------------------------------------|---------------------------------------|
| Gag | 89.6 | 93.7 |
| Pol | 94.9 | 95.9 |
| Env gp110 | 77.6 | 82.9 |
| Env gp41 | 85.5 | 90.1 |
| Tat | 78 | 88 |
| Rev | 84 | 88 |
| Vif | 89 | 92 |
| Nef | 78 | 91 |
| Vpu | 81 | 88 |

^a Alignments were performed according to the Wilburg and Lipman (1983) computer program, using a gap penalty of 1, K-Tuple of 1, and window of 20 aa.

^b HIV1-PRO and HIV1-NDK comparison.

^c HIV1-ELI and HIV1-NDK comparison.

(c) Sequence analysis of the HIV1-NDK genome

The complete nt sequence of HIV1-NDK is presented in Fig. 2 with the deduced aa sequence for all ORFs. The genomic organization of HIV1-NDK is similar to all other sequenced HIV1 strains. HIV1-NDK genome is 9143 nt long (in its RNA form) and includes three structural genes, *gag*, *pol* and *env*, and two transactivator genes, *tat* (Arya et al., 1985) and *rev* (Sodroski et al., 1985), each consisting of two coding exons (Sodroski et al., 1985, 1986b). The other regulatory genes, *vif* and *nef*, could also be detected. The HIV1-NDK sequence contains the gene *vpu*, which has been recently identified as a functional gene (Matsuda et al., 1988). No additional ORF, other than those present on other HIV1 strains, could be detected.

Percentage of aa homology was calculated for all ORFs of HIV1-NDK and HIV1 prototype (Table II). As expected, the highest genetic variability was observed for the ORF coding for Env. A comparison was also made with another Zaïrian strain, HIV1-ELI, previously sequenced (Alizon et al., 1986). This indicated that HIV1-NDK is more closely related to HIV1-ELI than to HIV1 prototype, in all ORFs (see

Table II, right side). In order to correlate these similarities with the biological function, HIV1-ELI strain was tested by MT4 assay. Our results (not shown) indicate that HIV1-ELI is 100 times less infectious and cytopathic than HIV1-NDK, and corresponds to an intermediate strain in terms of CPE.

The molecular basis of the high virulence of HIV1-NDK may originate from genetic changes occurring in several ORFs. To localize specific genetic differences between HIV1-NDK and other sequenced strains, alignments of all ORFs were performed. The hypervariable regions of Env of HIV1-NDK were localized as in other isolates. It was not surprising that maximum variability could be found in Env. Clusters of hypervariability are detected in the same position for HIV1 prototype, HIV1-ELI and another Zaïrian strain, HIV-Z6 (Srinivasan et al., 1987) (Fig. 3). The region of the Env located between aa 397 and 439 is responsible for binding with the CD4 molecule, and a stretch of 12 aa (410–421) plays a crucial role in this interaction (Lasky et al., 1987; Kowalski et al., 1987). These 12 aa are highly conserved in HIV1-NDK, which tends to indicate that the highly cytopathogenic properties of this strain

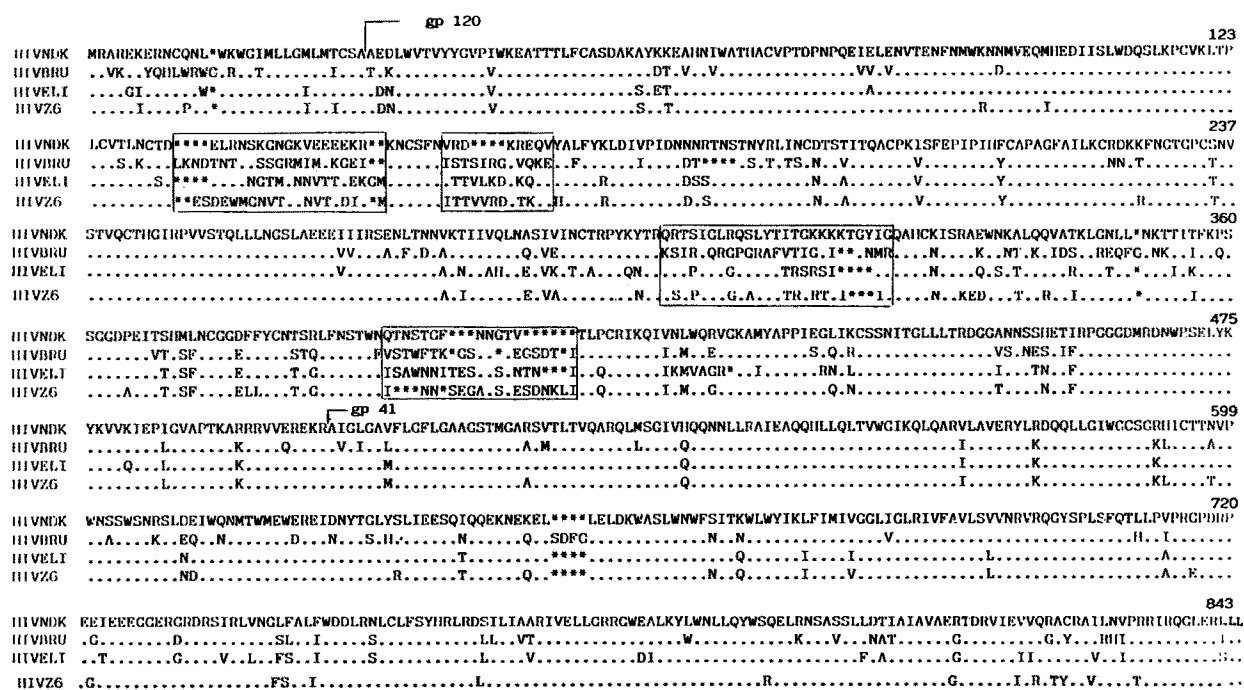


Fig. 3. Multi-alignment of envelope aa sequences from four viral isolates. HIV1-NDK was used as reference sequence. The minimum number of gaps was introduced. Boxes indicate hypervariable regions. Dots symbolize identical aa. Asterisks indicate deletions of aa. The border between gp120 and gp41 is shown by a bent line.

may not be due to a modification of the attachment site with CD4. It is possible that one or more of the hypervariable regions could react with the surface of the infected cell and enhance the CD4-Env interaction, which has been shown to play an important role in the cytopathogenicity (Sodroski et al., 1986a). The same type of hypervariable domains in other HIV1 variants could also be responsible for the induction of specific neutralizing antibodies against HIV1-NDK in some patients who did not develop HIV1-prototype-neutralizing antibodies (Rey et al., 1987). No doubt the relationships between structure and biological functions of hypervariable regions of different strains should be investigated further, since they have been associated with properties of such significance as attachment of the CD4 molecule, syncytia formation and cell fusion (Klatzman et al., 1984; Dagleish et al., 1986; Sodroski et al., 1986a; Kowalski et al., 1987).

A small deletion of 3 aa was detected at aa 122 of the Gag protein of HIV1-NDK (Fig. 4). This rather unusual feature was never observed in previously analysed sequences of protein P18 (Wain-Hobson et al., 1985). It should be noted that protein P18 represents the most external protein of the nucleocapsid: it is in contact with the transmembrane glycoprotein gp 41, as well as core proteins. Hence, a change in its spatial conformation might modify the spatial conformation of envelope proteins modulating binding to the CD4 receptor. The role of such a deletion remains unclear. The other deletion located in the 3' part of the *gag* gene is not HIV1-NDK specific, and has been found in other strains. Analysis of Pol protein did not show any evidence of specific differences.

In order to determine whether the high CPE could be explained by changes in the regulatory genes, comparisons of these regions were performed between HIV1-NDK and the prototype. The alignments of Rev, Vif, Nef and Tat proteins did not show any major differences, since point mutations were generally observed in all already known regions. However, the biological role of such variation cannot be excluded. In the case of Tat protein, all cysteine residues, which play an important role in its function (Frankel et al., 1988), were conserved in both strains. The LTRs of HIV1-NDK and HIV1 prototype share 90% of nt homology. The Tar region (nt -17 to +54), which is required in *cis* for *trans*-

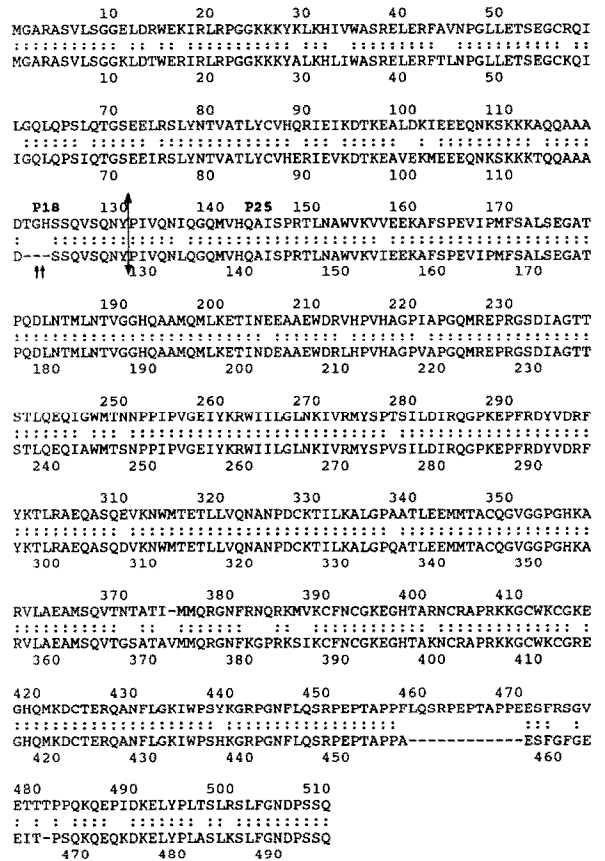


Fig. 4. Alignment of Gag aa sequences of HIV1 prototype and HIV1-NDK. Double dots indicate identical aa. Dashes indicate deletions of aa. The predicted cleavage site between P18 and P25 is symbolized by the vertical arrow. The specific deletion observed at the C-terminal portion of HIV1-NDK P18 protein is indicated by the two small upward arrows. Alignment was performed according to Wilburg and Lipman (1983) parameters, using a gap penalty of 1, K-Tuple of 1 and window of 20 aa.

activation by Tat (Rosen et al., 1985), is highly conserved, with 68 out of 71 nt being homologous. The other regions implicated in transcriptional regulation, the TATA box (nt -23 to -28) (Varmus, 1982) and the target sequence for Sp1 cellular factor (nt -43 to -85) (Jones et al., 1986), are also very similar. The enhancer region (nt -80 to -105), which is the target sequence for NF-KB transcription factor (Nabel and Baltimore, 1987), is exactly the same for both HIV1-NDK and HIV1 prototype. All the differences were found upstream from nt -160, where the *cis*-acting element responsive to *nef* might be located (Ahmad and Venkatesan, 1988). It is tempting to correlate these nt variations with a dysfunction of HIV1-NDK negative regulation, which also could be related to high CPE.

(d) Conclusions

To determine specific genetic differences involved in the highly cytopathic properties of the HIV1-NDK isolate, 9143 nt have been sequenced. The analysis of the sequences shows that some minor molecular differences could be responsible for the increase in virulence. They could be located in either the U3 part of the LTR, or in the C-terminal part of P18, or associated with a hypervariable domain of Env. Experiments to determine which of these molecular differences is related to the high CPE of HIV1-NDK are now in progress.

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