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Generation of the Replication-Competent Human Immunodeficiency Virus Type 1 Which Expresses a Jellyfish Green Fluorescent Protein

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The gene encoding a red-shifted green fluorescent protein variant (EGFP) was introduced into a human immunodeficiency virus type 1 (HIV-1) molecular clone by replacing the *nef* gene. The EGFP-expressing HIV-1 replicated efficiently in established human T cells. The expression of EGFP in the virus-infected cells was confirmed by fluorescence microscopy and also by Western blot analysis using the GFP antibodies. The EGFP gene was stably maintained in the viral genome during prolonged passages. EGFP-expressing HIV-1 could be used for anti-HIV-1 drug screening, sorting of virus-infected cells by fluorescence activated cell sorting (FACS) analysis, and for the rapid and simple detection of virus-infected cells by fluorescence microscopy.

Infection with human immunodeficiency virus type 1 (HIV-1) causes progressive T cell loss and the consequent acquired immunodeficiency syndrome (AIDS) [1]. Extensive efforts have been devoted to understand the pathogenic mechanism of HIV-1 [2]. The HIV-1 clones which express marker genes such as chloramphenicol acetyltransferase [3] and firefly luciferase genes [4] have been developed. These marker genes were able to facilitate the detection of HIV-1 infection at high sensitivity by simple enzyme assays. These recombinant viruses were suggested to be used to trace the route of infection following the infection of an experimental animal.

The green fluorescent protein (GFP) from jellyfish is a recently developed marker for the gene transfer [5]. GFP emits green light when illuminated by the blue or UV light [6]. The fluorescence of GFP is its intrinsic property and does not require any cofactors and substrates, thereby allowing its detection in living cells [7-13]. The GFP chromophore is produced by autocyclization of three residues serine-65, dehydrotyrosine-66 and glycine-67 [8]. The GFP chromophore is extremely stable and resistant to photobleeching, allowing detection over a long time [5]. EGFP, a red-shifted GFP variant, contains the two amino acid substitution mutations of F64L and S64T which increase the fluorescence intensity by 35 folds [14]. Furthermore, EGFP contains more than 190 silent base mutations which create an open reading frame composed of preferred human codons and ensure the maximal mammalian expression [14].

In this work, we constructed and characterized the replication-competent HIV-1 clone which expresses EGFP. The EGFP gene was introduced to the HIV-1 genome in place of *nef*. The EGFP-expressing HIV-1 produced bright green fluorescence in the infected cells, implicating its value as a tool to study the infective process of HIV-1.

MATERIALS AND METHODS

Plasmid construction. pNL-3′ plasmid was constructed by inserting the region between *Bam*HI (8465) and *Nco*I (flanking sequence) sites of the HIV-1 clone pNL43 [15] into the pSK-Bluescript (Stratagene) vector which was cut by *Bam*HI and *Nae*I. The region between *Bam*HI (8465) and *Kpn*I (9005) of pNL-3′ was replaced with a 321 bp DNA fragment which was generated by PCR amplification using the template pNL-3′ and the primers (T3 and 8786-kpn1), followed by digestion with *Bam*HI and *Kpn*I. The sequence of 8786-kpn1 primer is 5′-GCCAGGTACCTTATAGCAAAATCCT-3′, (*Kpn*I recognition sequence is underlined). The resulting NL-3△nef was digested with *Asp*718, treated with the Klenow enzyme, and then ligated with the 745 bp *Bam*HI-*Not*I fragment of pEGFP-1 (Clontech) which was blunt-ended with the Klenow enzyme to produce NL-3′EGFP. NL-EGFP was constructed by inserting the *Bam*HI-*Nco*I fragment of NL-3′EGFP into pNL43 which was cut by the same restriction enzymes.

Cell culture and transfection. Sup T1 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. Plasmid transfections were carried out by the DEAE-dextran method [16]. Five million Sup T1 cells were transfected with each 5 μg of plasmids.

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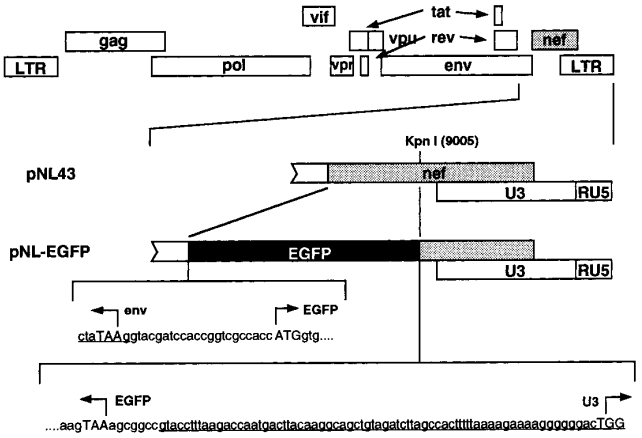


FIG. 1. Schematic representation of wild type and the EGFP-expressing HIV-1 clone. The 3′ region of the viral genome was described in detail. The nucleotide sequences around the junction sites between EGFP gene and the viral genome are described. The nucleotide sequences originated from the viral genome are underlined. The start and the termination codons of *env* and EGFP genes and the nucleotides at the start site of U3 are shown in uppercase.

Reverse transcriptase assay. The virus particles in the culture supernatant were precipitated by centrifugation at 800xg for 40 minutes in 15% polyethyleneglycol/0.7 M NaCl solution. The reverse transcriptase activities were determined with the Reverse Transcriptase Assay, non-radioactive kits (Boehringer Mannheim) according to the manufacturer's instruction.

Fluorescence microscopy. Cells were visualized under the IMT-2 inverted microscope equipped with a blue light excitation filter (Olympus) and photographed with a Kodak (400 ASA) film.

Immunoblots. Proteins were separated on a 10% denaturing polyacrylamide gel, and then blotted to a Hybond-ECL nitrocellulose membrane (Amersham). ECL Western blotting was performed according to the manufacturer's instruction with HIV-1 immune globulin or with the GFP polyclonal antibody (Clontech). The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV immune globulin (human) from Dr. A. Prince.

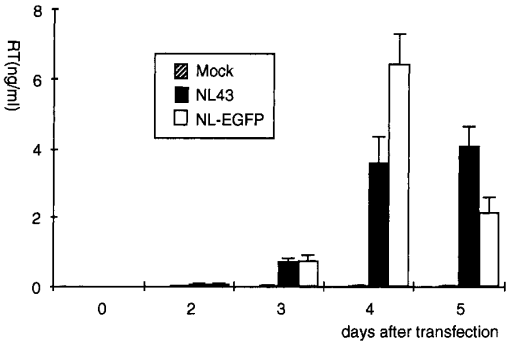
RESULTS AND DISCUSSION

The structure of pNL-EGFP plasmid is schematically drawn in Figure 1. To replace the *nef* gene of HIV-1 with EGFP, the translational start codon of *nef* was destroyed and a *Kpn*I site was generated at the 8786th nucleotide position. EGFP gene of 745 bp was inserted between the two *Kpn*I sites, resulting in a net size increase of 530 bp of the viral genome over the wild type.

It was proposed that the foreign gene inserted into HIV-1 provirus interfered with the viral replication [3]. Authors showed that a net size increase of as little as 570 bp by inserting the CAT gene into HXBc2 strain of HIV-1 deteriorated the viral replication, even only slightly. To test whether the insertion of EGFP gene affected the viral replication, the wild type pNL43 and pNL-EGFP plasmid was transfected into Sup T1 cells (Fig. 2). The production of virion particles was determined by assaying the reverse transcriptase activity in the culture supernatant and observing the syncytia formation of the infected cells. NL-EGFP virus replicated as efficiently as and a little faster than the wild type virus. Furthermore, the viral gene expression profile of NL-EGFP virus was exactly the same as that of the wild type virus (Fig. 3A). These results suggest that there are little size constraints for the viral replication by insertion of EGFP gene.

In order to determine that EGFP protein was efficiently produced from the NL-EGFP-infected cells, Sup T1 cells were infected with the wild type or NL-EGFP virus. EGFP protein in the cell extract was identified by Western blot analysis using the polyclonal antibody against EGFP protein (Fig. 3B). NL-EGFP produced a single band of 27 kD molecular mass which corresponds to the EGFP protein. Virus-infected cells were also examined under the fluorescence microscope to confirm the expression of EGFP gene. Sup T1 cells infected with the wild type virus showed only dark background, when illuminated with the blue light (Fig. 3C). On the other hand, bright green fluorescence could be easily observed in cells infected with the recombinant NL-EGFP virus (Fig. 3D). NL-EGFP virus also replicated efficiently and produced bright fluorescence in Jurkat

(A)



(B) Syncytia formation

	day0	*day2*	*day3*	*day4*	*day5*
Mock	-	-	-	-	-
NL43	-	-	+	++	++++
NL-EGFP	-	+	++	+++	++++

FIG. 2. *In vitro* replication of the EGFP-expressing HIV-1. Five millions Sup T1 cells were transfected with 5 μg of wild type or EGFP-expressing clone. The reverse transcriptase activities in the culture supernatants (A) and syncytia formation of the cells (B) were determined according to the time course. − : no syncytia formation; + : less than 5% cells form syncytia: ++: 5∼30% cells form syncytia; +++ : 30∼70% cells form syncytia; ++++ : more than 70% cells form syncytia.

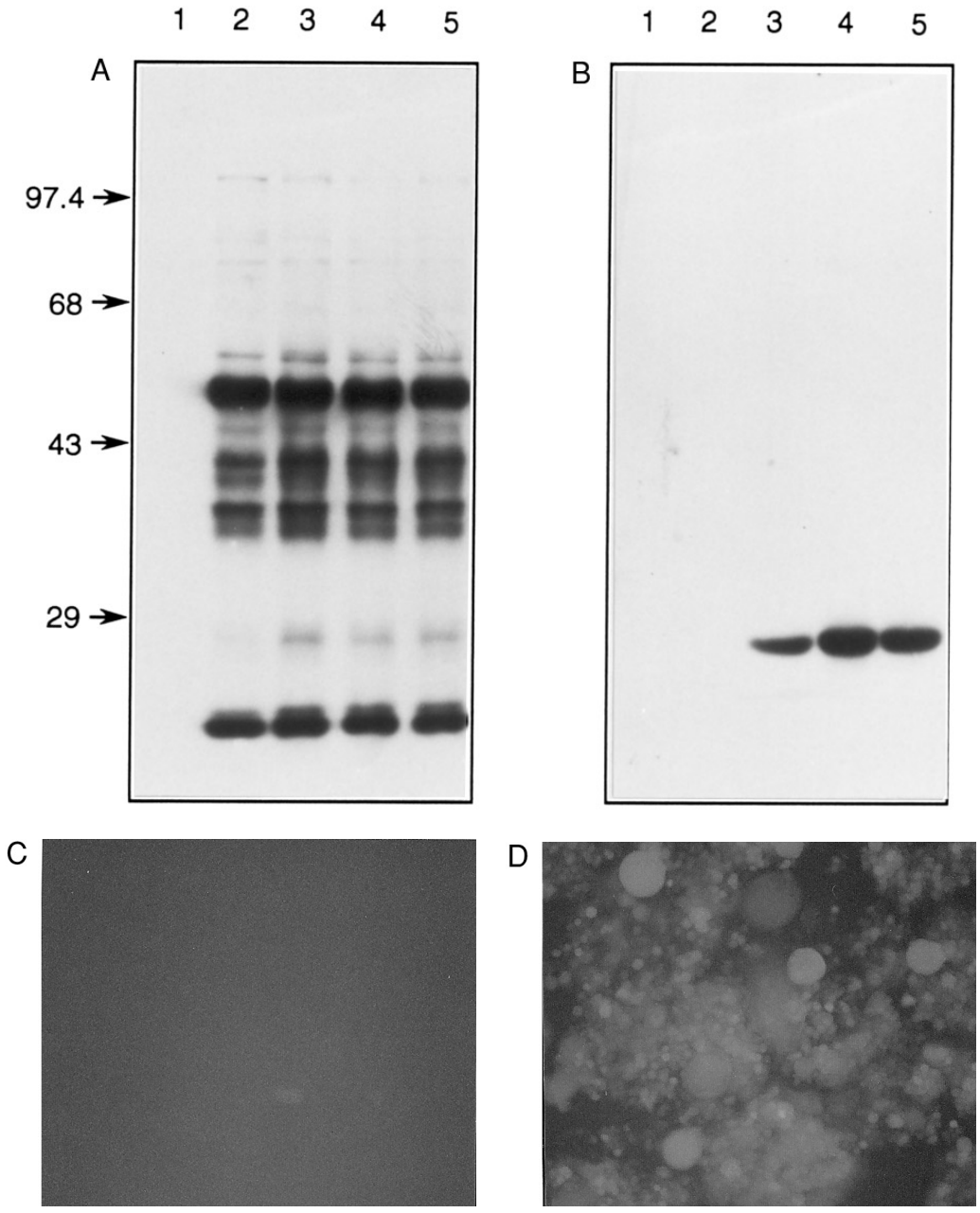


FIG. 3. Detection of EGFP in virus-infected cells. Virus-infected cells were analyzed for the expression of viral proteins (A) and EGFP (B) by ECL Western blotting using HIV-1 positive sera and GFP polyclonal antibody (Clontech), respectively. Lane 1, Mock-infected; lane 2, NL43-infected; lane 3, NL-EGFP-infected (passage 1); lane 4, NL-EGFP-infected (passage 5); lane 5, NL-EGFP-infected (passage 10). The migration of the molecular weight standards (kDa) is indicated. NL43 (C) and NL-EGFP-infected Sup T1 cells (D) were examined under the fluorescence microscope and then photographed with Kodak (400 ASA) film.

cells (data not shown). These results showed that EGFP protein was efficiently produced from the NL-EGFP-infected cells, and its fluorescence could be used as a marker for the infection of recombinant viruses.

The recombinant NL-EGFP virus was analyzed for the genomic stability after serial passages in Sup T1 cells. Briefly, one million Sup T1 cells were infected with the culture supernatant containing 100 pg of reverse transcriptase. After five days, the culture supernatant was used to reinfect fresh cells. When NL-EGFP virus-infected cells after the 5th and 10th passage were analyzed, no significant reduction of the expression level of viral and EGFP genes was detected (Fig 3A and 3B, lane 4 and 5). Furthermore, cells infected with

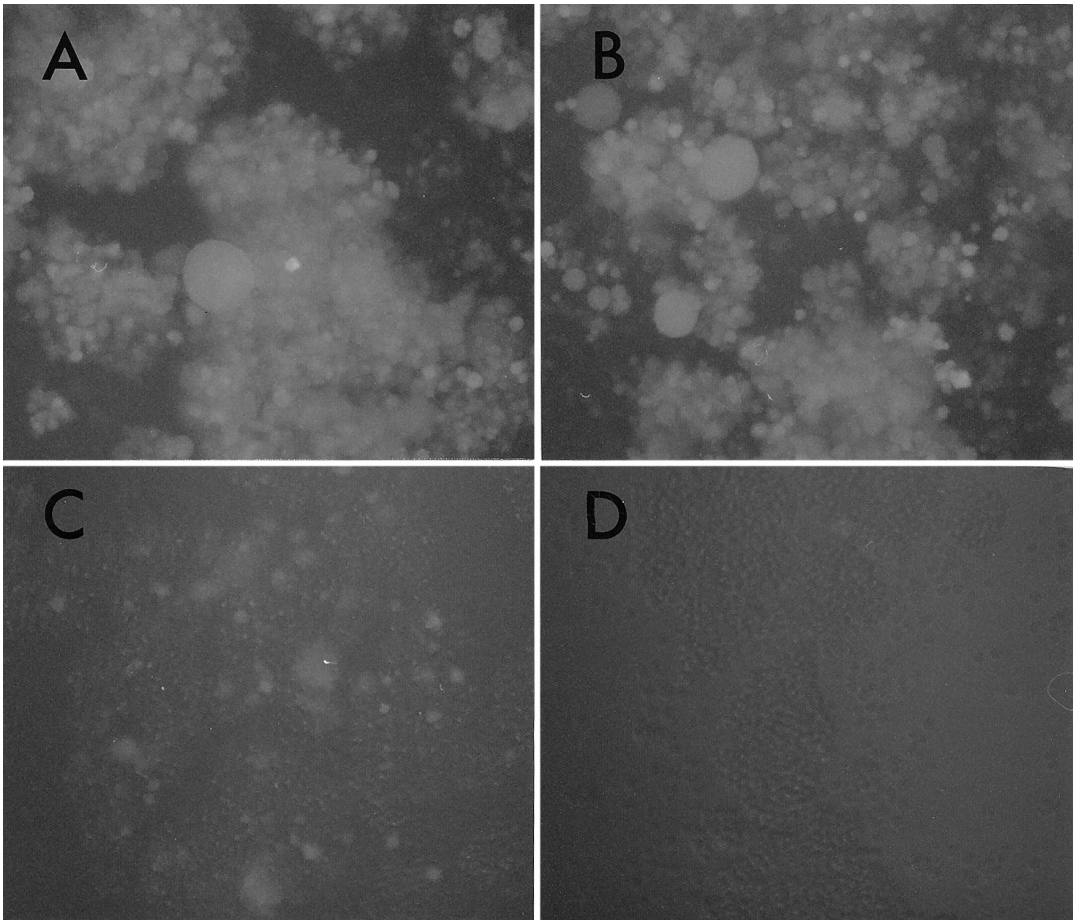


FIG. 4. Inhibition of viral replication by azidothymidine (AZT). 2×10^5 Sup T1 cells were infected with 10 pg of RT units and put into each wells of a 24-well plate. AZT of various concentrations was added to each well. The AZT concentration in the culture medium was (A) 0 nM; (B) 1 nM; (C) 10 nM; (D) 1 μM. The cells were subcultured every two days with the AZT-containing medium. Six days after the infection, cells were visualized under the fluorescence microscope and then photographed.

the NL-EGFP virus from 10th passage was shown to emit the green fluorescence light with the intensity comparable to the cells from the first passage (data not shown). These results suggested that NL-EGFP viruses stably accommodated EGFP gene, even though the viral genome was enlarged by as many as 530 bp.

We tested one of the possible applications of NL-EGFP in that it could be used for the anti-HIV-1 drug screening. Various concentrations of azidothymidine (AZT) were treated to Sup T1 cells infected with NL-EGFP virus. Six days after the infection, cells were observed under the fluorescence microscope (see Fig. 4). The fluorescence intensities and the number of the cells emitting green fluorescence were proportionally decreased as the AZT concentrations were increased. The difference of the fluorescence intensity well agreed with other virus quantification methods such as syncytia formation and reverse transcriptase assays. These results indicated that NL-EGFP virus would be useful for the screening of the anti-HIV-1 drugs.

EGFP is a humanized red-shifted variant with two amino acid substitution in the chromophore [14]. These optimizations facilitate the detection of EGFP at higher sensitivity. Unlike NL-EGFP, NL-GFP which had wild type GFP in place of *nef* showed undetectable level of fluorescence (data not shown).

EGFP, as a marker for the viral infection, has several advantages comparing with the previously reported CAT [3] and luciferase [4]. First, virus-infected cells could be simply identified by observing the cells under the fluorescence microscope. Second, EGFP enables the identification of the virus-infected cells without killing them. Third, virus-infected cells could be readily separated by fluorescence activated cell sorting (FACS) analysis. Fourth, many current diagnostic procedures of HIV-1 such as p24 and viral reverse transcriptase assays quantitate specific viral proteins, not infectious virus particles. Thus, these methods appear to bear the risk of an error in a measurement of infectious virus titer by overproduction of defective virus, which is a

general phenomenon of HIV-1 [17]. In this regard, our NL-EGFP system has an advantage in terms of in vivo detection of infectious virus indirectly. These features implicate that our EGFP-expressing HIV-1 could also be useful to study the infective process of HIV-1.

ACKNOWLEDGMENTS

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