研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
T. Hagiwara, J. Hattori, T. Kaneda.	PNA-In Situ Hybridization Method for Detection of HIV-1 DNA in Virus-Infected Cells and Subsequent Detection of Cellular and Viral Proteins.	I. A. Darby	In Situ Hybridization Protocols 3rd edition	Humana Press	NJ	139-149	2006

雑誌

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M. Takahashi, S. Ibe, Y. Kudaka, N. Okumura, A. Hirano, T. Suzuki, N. Mamiya, M. Hamaguchi and T. Kaneda.	No observable correlation between central nervous system side effects and EFV plasma concentration in Japanese HIV-1 infected patients treated with EFV containing HAART.	AIDS Res. Hum. Retroviruses			in press

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Ⅳ. 研究成果の刊行物・別刷

9

PNA–*In Situ* Hybridization Method for Detection of HIV-1 DNA in Virus-Infected Cells and Subsequent Detection of Cellular and Viral Proteins

Tomoko Hagiwara, Junko Hattori, and Tsuguhiro Kaneda

Summary

We describe *in situ* hybridization protocols using peptide nucleic acid (PNA) as a probe for detecting HIV-1 DNA in virus-infected cells and the subsequent detection of cellular and/or viral proteins. Because a PNA probe of approx 20 bases was sufficiently long to detect a specific target sequence, a conserved sequence of such a short length was easily identified. Therefore, this probe is valuable even to identify quasi-species of HIV-1. In addition, we adopted a catalyzed signal amplification method to amplify weak viral DNA signals; thus, stringent washing was crucial for eliminating false-positive signals. Our double-staining method using PNA–*in situ* hybridization and subsequent immunostaining enabled the active and inactive proviruses to be distinguished.

Key Words: *In situ* hybridization; peptide nucleic acid; catalyzed signal amplification; HIV-1 provirus; CD4-positive T lymphocytes; p24; HLA-DR.

1. Introduction

In situ hybridization (ISH) is now popularly used in cytogenetic studies to determine the localization of a specific gene on a chromosome and to detect mRNA expression and viral infection within cells using DNA or RNA probes. Nonradioactive ISH methods using fluorescence or visible light to visualize signals generally are used for the detection of target nucleic acid sequences. In general, probes of more than 500 bp in length are required in such studies *(1)*.

Until recently, the detection of HIV-1 has been performed by using autoradiography, using probes labeled with radioisotopes, such as ^{35}S *(2–4)* and ^{125}I *(2,5)*. Although the use of radioactive ISH for the detection of HIV-1 is time consuming and not very convenient, the small copy number of HIV-1 in

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infected cells has hindered the development of a conventional nonradioactive detection system. In addition, designing a suitable proper probe with a length of more than 150 bp is very difficult because of the general lack of long-conserved DNA sequences in viruses, such as HIV-1, that have error-prone reverse transcriptase without any repair activity. To bypass this problem, we developed a peptide nucleic acid (PNA) that mimics the DNA configuration *(6–8)*. PNA, being electrically neutral, can penetrate into cells more easily than DNA and, in addition, PNA can more strongly hybridize with DNA than DNA *(9,10)*. For these reasons, a PNA probe of approx 20 bases in length is long enough to detect a specific target sequence *(11)*.

To overcome the weak viral DNA signal, one of the following methods for signal amplification can be used: one is *in situ* polymerase chain reaction (PCR *[12]*), and the other is a catalyzed signal amplification (CSA) using biotinyl tyramide *(13)*. *In situ* PCR is quite powerful for amplification of rare target DNA within cells; therefore, *in situ* PCR-driven ISH would be suitable for detecting low copy number DNA sequences. However, this method carries the risk of amplicons synthesized *in situ* diffusing and resulting in false-positives *(14)*. On the other hand, a single copy of the HPV-16 virus was detected successfully using the CSA method *(14)*. Therefore, we adopted the CSA method for detecting HIV-1 DNA *(15)* and incorporated a crucial stringent washing step to eliminate nonspecific signals that arise from the CSA procedure.

Here, we describe the procedures for detecting HIV-1 DNA in infected CD4-positive T lymphocytes, and the phenotypic determination of HIV-1 DNA-positive cells by a double staining method.

2. Materials

2.1. Cell Lines

1. ACH2: positive control. Human lymphoid cell line latently infected with HIV-1.
2. MOLT4-IIIB: positive control. Human leukemic cell line persistently infected with HIV-1 strain IIIB.
3. MOLT4: negative control.

2.2. Sample Preparation

2.2.1. CD4-Positive T-Lymphocytes Smears

1. StemSep column chromatography (Stem Cell Technologies, Inc., Vancouver, BC, Canada; cat. no. STS-14032).
2. Phosphate-buffered saline (PBS) with 3% fetal bovine serum.
3. 4% paraformaldehyde (PFA) containing 0.1 *M* sodium phosphate buffer, pH 7.4.
4. Ethanol.
5. Silane-coated slides.

6. Wax pen (DakoCytomation A/S, Glostrup, Denmark).
7. Staining racks and containers.
8. Dryer.

2.2.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Tissue blocks of 20% formalin-fixed or 4% PFA-fixed, paraffin-embedded samples.
2. Silane-coated slides.
3. Staining racks and containers.
4. Xylene.
5. Rehydration series: 100, 95, 90, and 70% ethanol.

2.3. Pretreatment

2.3.1. CD4-Positive T Lymphocytes Smears

1. Tris-buffered saline containing Tween-20 (TBST): 50 m*M* Tris-HCl, 300 m*M* NaCl, 0.1% Tween-20, pH 7.6.
2. Target retrieval solution (DakoCytomation A/S; cat. no. S1700).
3. Methanol containing 0.3% H_2O_2.
4. Water bath.
5. Staining racks and plastic containers.

2.3.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. The same as **Subheading 2.3.1., items 1–5**.
2. Proteinase K (DakoCytomation A/S, S3004).

2.4. Preparation of PNA Probe

1. PNA probe: the structure of the probe is as follows: FITC-HN-GCAGCTTCCT-CATTGATGG-$CONH_2$ (FASMAC Co. Ltd., Kanagawa, Japan; *see* **Note 1**).
2. DNA ISH solution (DakoCytomation A/S; cat. no. S3305).
3. Cover slips.

2.5. Heat Denaturation

1. Hotplate.

2.6. Hybridization

1. Stringent wash solution (DakoCytomation A/S; cat. no. K5201).
2. TBST.
3. Incubator.
4. Plastic containers.
5. Water bath.
6. Moist chamber.

2.7. PNA Probe Detection by CSA

1. Horseradish peroxidase (HRP)-conjugated anti-FITC antibody (DakoCytomation A/S; cat. no. P5100).
2. Biotinyl tyramide solution (DakoCytomation A/S, GenPoint kit K0620).
3. HRP-conjugated streptavidin (DakoCytomation A/S, GenPoint kit K0620).
4. Alexa Fluor 488-labeled streptavidin (Invitogen, Carlsbad, CA).
5. 4,6-diamino-2-phenylindole (DAPI) II (Vysis, Inc., Downers Grove, IL).
6. TBST.
7. Staining racks and containers.
8. Cover slips.

2.8. PNA–ISH and Immunohistochemistry (IHC) (Indirect Method)

1. Mouse anti-human CD4 monoclonal antibody (Novocastra Laboratories, Ltd., Newcastle, UK; cat. no. NCL-CD4-1F6) or mouse anti-human HLA-DR monoclonal antibody (DakoCytomation A/S; cat. no. M0746).
2. Alexa Fluor 594-labeled goat anti-mouse IgG antibody (Invitrogen; cat. no. A-11005).
3. DAPI II (Vysis, Inc.).
4. Cover slips.

2.9. PNA–ISH and IHC (Labeled Streptavidin-Biotin Method)

1. Avidin solution (DakoCytomation A/S; cat. no. X0590).
2. Biotin solution (DakoCytomation A/S; cat. no. X0590).
3. Mouse monoclonal anti-HIV-1 p24 antibody (DakoCytomation A/S; cat. no. M0857).
4. Biotinylated goat anti-mouse Ig antibody (DakoCytomation A/S; cat. no. E0433).
5. Alexa Fluor 594-labeled streptavidin (Molecular Probes, Inc.; cat. no. S-11227).
6. DAPI II (Vysis, Inc.).
7. Cover slips.

3. Methods

3.1. Sample Preparation (see Note 2)

3.1.1. CD4-Positive T Lymphocytes Smears

1. Negatively select and purify CD4-positive T lymphocytes by StemSep column chromatography according to the manufacturer's instructions.
2. Spin down the collected cells at 250*g* for 5 min.
3. Discard supernatant and resuspend with PBS.
4. Mark the area for sample-mount on a silane-coated slide with a wax pen. Drop 5 µL of the cell suspension onto the slide and spread it out gently using the pipet tip.
   The area of the sample-mount (15 × 15 mm).
5. Dry the slides using a dryer at a cool setting.
6. Fix the slides with 4% PFA containing 0.1 mol/L sodium phosphate buffer, pH 7.4, at room temperature for 60 min or at 4°C overnight.

7. Rinse the slides in PBS (3 min, three times).
8. Dehydrate the slides in absolute ethanol and then store at –20°C until use.

3.1.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Place a 4- to 5-µm section onto a slide. Heat the slide to melt the paraffin in a 60°C oven for 15 min and dry at 37°C overnight.
2. Deparaffinize sections in fresh xylene (3 min, three times) and rehydrate in graded (100, 100, 95, 90, and 70%) ethanols and autoclaved water.

3.2. Pretreatment

3.2.1. CD4-Positive T-Lymphocyte Smears

1. Immerse the slides in autoclaved water for 5 min.
2. Immerse the slides in preheated target retrieval solution for 40 min at 95°C, and allow to cool for 20 min.
3. Wash the slides in autoclaved water (1 min, three times).
4. Immerse the slides in methanol containing 0.3% H_2O_2 for 20 min.
5. Wash the slides in autoclaved water for 1 min.
6. Briefly immerse slides in 95% ethanol and allow to air dry.

3.2.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Immerse the slides in autoclaved water for 5 min.
2. Immerse the slides in preheated target retrieval solution for 40 min at 95°C and allow to cool for 20 min.
3. Wash the slides in autoclaved water (1 min, three times).
4. Digest sections with proteinase K for 10 min at room temperature (*see* **Note 3**).
5. Wash the slides in autoclaved water (1 min, three times).
6. Treat the slides with methanol containing 0.3% H_2O_2 for 20 min.
7. Wash the slides in autoclaved water for 1 min.
8. Briefly immerse the slides in 95% ethanol and allow to air-dry.
9. Mark the area of the section with a wax pen.

3.3. Preparation of PNA Probe

1. Dilute FITC-conjugated PNA probe in hybridization solution to a final concentration of between 0.2 and 0.5 µg/mL.
2. Apply 25 µL of hybridization solution containing PNA probe to the marked area of the slide.
3. Carefully apply the cover slip, avoiding the introduction of air bubbles.

3.4. Heat Denaturation

1. Heat the slides at 93°C for 5 min on a hotplate to denature the double-stranded DNA.

3.5. Hybridization

1. Incubate the slides with the PNA probe at 45°C for 60–90 min in a moist chamber.
2. After hybridization, immerse the slides in TBST and gently remove the cover slips.

3. Wash the slides in prewarmed stringent wash solution at 57°C (20 min, twice).
4. Immerse the slides in TBST at room temperature for 5 min.

3.6. PNA Probe Detection by CSA (see Notes 4 and 5; Fig. 1)

1. Incubate HRP-conjugated rabbit anti-FITC antibody (1:500 dilution) for 60 min (*see* **Note 6**).
2. Wash the slides in TBST (3 min, three times).
3. Incubate biotinyl-tyramide for 15 min.
4. Wash the slides in TBST (3 min, three times).
5. Incubate HRP-conjugated streptavidin (1:600–800 dilution) for 15 min.
6. Wash the slides in TBST (3 min, three times).
7. Incubate biotinyl-tyramide for 15 min.
8. Wash the slides in TBST (3 min, three times).
9. Incubate 0.5 µg/mL Alexa Fluor 488-labeled streptavidin for 15 min in the absence of light.
10. Wash the slides in TBST in the absence of light (3 min, three times).
11. Immerse the slides in distilled water.
12. Apply DAPI II and mount cover slip.

3.7. Fluorescence Microscopy

The slides were examined under a fluorescence microscope (BX50 and BX-FLA, Olympus Corp., Tokyo, Japan) equipped with appropriate filter sets (61002, Chroma Technology Corp., Rockingham, VT). Photographic images of the fluorescent signals were taken with a CCD camera (SenSys 0400, Photometrics Ltd., Tucson, AZ) and were uploaded to a microcomputer using IPLab software (Scanalytics, Inc., Fairfax, VA). The stored images were merged to reveal various aspects (*see* **Note 7**; **Fig. 2**).

3.8. Subsequent Determination of Phenotypes After PNA–ISH Treatment

We describe here a double staining method by PNA–ISH and IHC methods for the detection of both HIV-1 DNA and a protein in the same cell, respectively. Proteins can be detected with one of two methods with IHC: an indirect method and a labeled streptavidin-biotin (LSAB) method. For the detection of CD4 or HLA-DR molecules, the conventional indirect method was used. On the other hand, the LSAB method that has higher sensitivity than the indirect method was performed to detect the p24 HIV-1 capsid protein.

Fig. 1. (*opposite page*) Schematic representation of the procedures for using the PNA-probe in the ISH method. The hybridized probe was detected by sequential reactions of the following antibodies and reagents: HRP-conjugated anti-FITC antibody, biotinylated tyramide (first amplification), HRP-labeled streptavidin, biotinylated tyramide (second amplification), and streptavidin-conjugated Alexa 488.

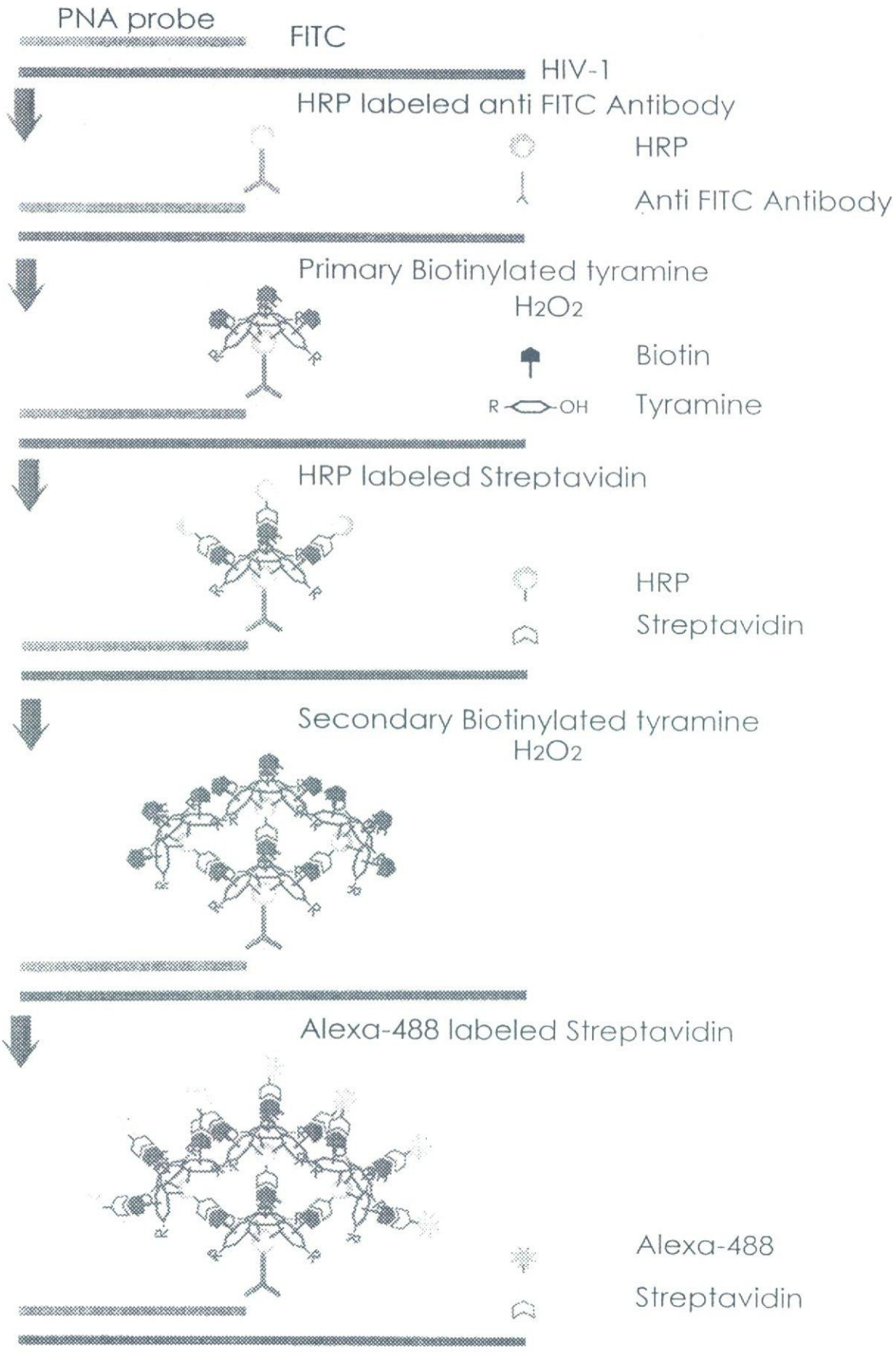
PNA probe
FITC
HIV-1
HRP labeled anti FITC Antibody
HRP
Anti FITC Antibody
Primary Biotinylated tyramine
H2O2
Biotin
R OH
Tyramine
HRP labeled Streptavidin
HRP
Streptavidin
Secondary Biotinylated tyramine
H2O2
Alexa-488 labeled Streptavidin
Alexa-488
Streptavidin

Fig. 1

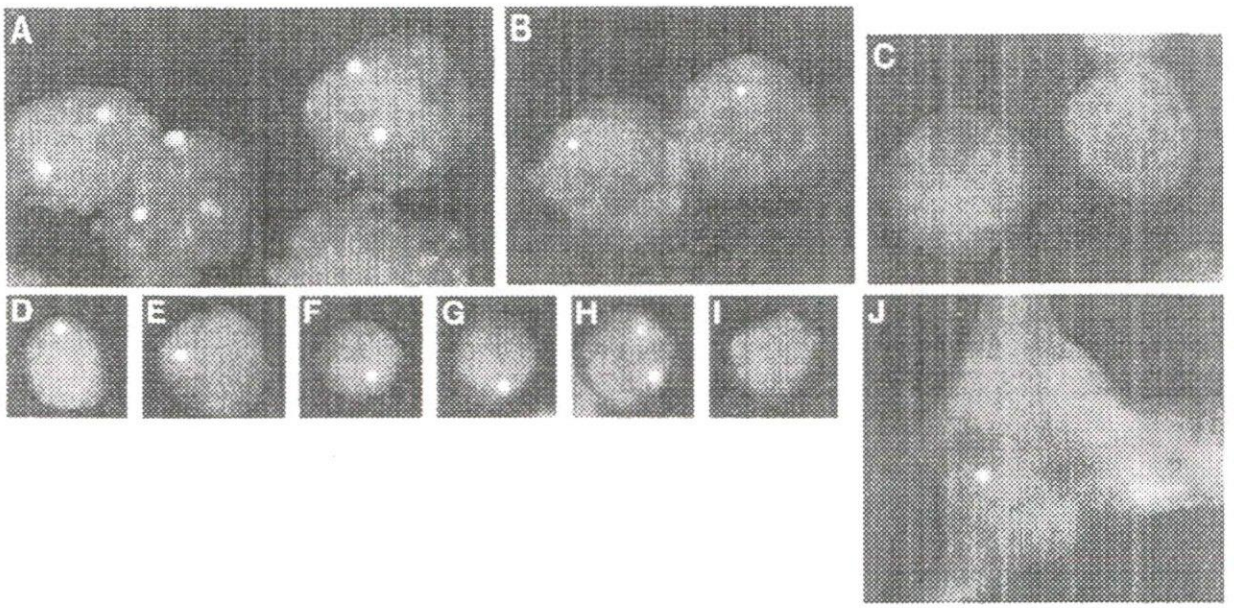

Fig. 2. Detection of HIV-1 provirus by PNA–ISH. Signals detecting HIV-1 proviruses were observed as green dots in a nucleus stained blue by DAPI. (**A**) MOLT4-IIIB. One to four proviruses per cell. (**B**) ACH2. One provirus per cell. (**C**) Negative control (MOLT4). No provirus. (**D–H**) CD4-positive T lymphocytes from HIV-1-infected patients. Most provirus-positive cells contained one provirus. Positive cells rarely contained more than two proviruses. (**I**) Negative control (CD4-positive T lymphocytes from a HIV-1-negative volunteer). No provirus. (**J**) Paraffin-embedded section of bone marrow from an AIDS patient. Positive cells contained one provirus in a nucleus.

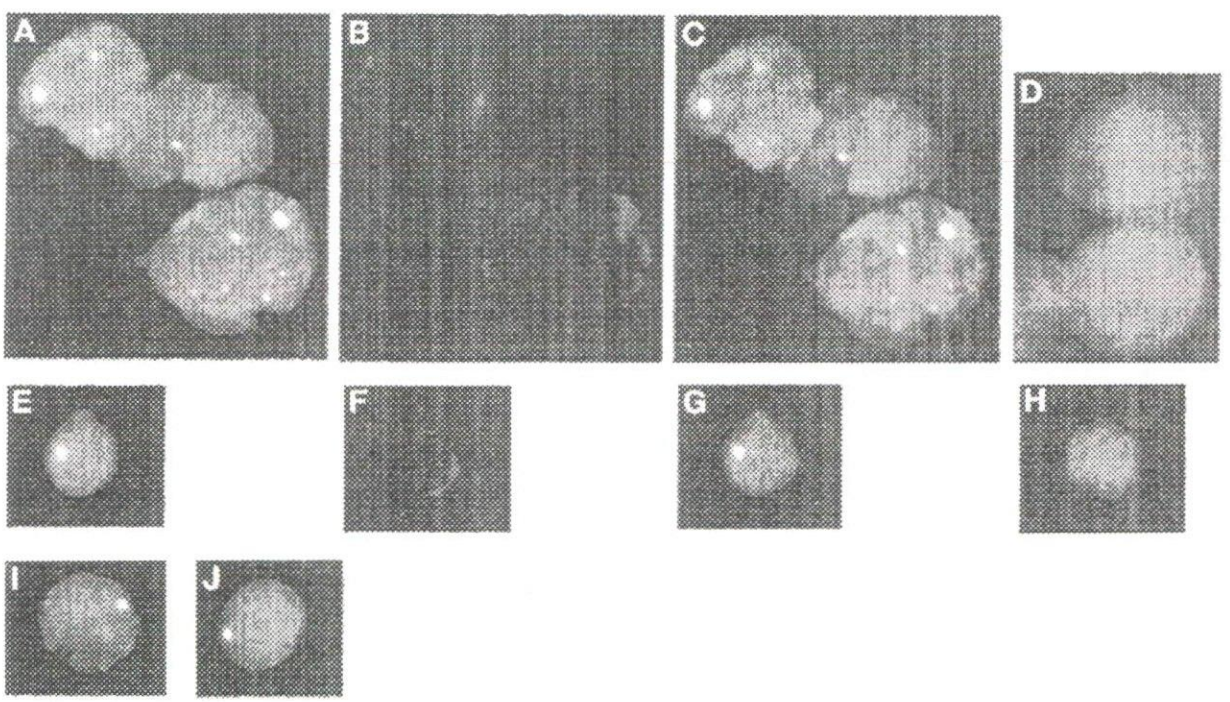

Fig. 3. Subsequent determination of phenotypes after PNA–ISH treatment. Proteins were stained red and localization of the proteins can be observed. (**A–C**) p24/HIV-1 provirus of MOLT4-IIIB. (**D**) Negative control (MOLT4). (**E–G**) p24/provirus

3.8.1. PNA–ISH and IHC (Indirect Method)

1. Follow **steps 1–10** inclusive for PNA Probe Detection by CSA (**Subheading 3.6.**).
2. Incubate with mouse anti-human CD4 monoclonal antibody or mouse anti-human HLA-DR monoclonal antibody at 4°C overnight.
3. Wash in TBST (3 min, three times).
4. Incubate with Alexa Fluor 594-labeled goat anti-mouse IgG antibody for 30 min.
5. Wash in TBST (3 min, three times).
6. Apply DAPI II and mount a cover slip.

3.8.2. PNA–ISH and IHC (LSAB Method)

Biotin blocking of biotinyl-tyramide after PNA–ISH staining is recommended before the reaction with primary antibody for the target protein.

1. Follow **steps 1–10** inclusive for PNA Probe Detection by CSA (**Subheading 3.6.**).
2. Incubate with avidin for 10 min.
3. Wash in TBST (3 min, three times).
4. Incubate with biotin for 10 min.
5. Wash in TBST (3 min, three times).
6. Incubate with mouse monoclonal anti-HIV-1 p24 antibody at 4°C overnight.
7. Wash in TBST (3 min, three times).
8. Incubate with biotinylated goat anti-mouse Ig antibody for 30 min.
9. Wash in TBST (3 min, three times).
10. Incubate with Alexa Fluor 594-labeled streptavidin for 15 min.
11. Wash in TBST (3 min, three times).
12. Apply DAPI II and mount a cover slip.

3.8.3. Fluorescence Microscopy of Double-Stained Sample

The slides were examined under a fluorescence microscope with appropriate filter sets. Photographic images of the fluorescent signals were taken with a CCD camera, and were uploaded to a microcomputer using IPLab software. The stored images were merged to reveal various aspects (**Fig. 3**).

Fig. 3. *(continued from opposite page)* of CD4-positive T lymphocytes from HIV-1-infected patients. (**H**) Negative control (CD4-positive T lymphocytes from a HIV-1-negative volunteer). (**A**,**E**) provirus (green) and nucleus (blue) were merged. (**B**,**F**) p24 (red). p24 HIV-1 capsid proteins were observed in the cytoplasm of HIV-1 provirus positive cells. (**C**,**G**) provirus (green), p24 (red) and nucleus (blue) were merged. (**I**,**J**) CD4-positive T lymphocytes from a HIV-1-infected patient; HIV-1 provirus (green) was seen in the cell nucleus. In contrast, CD4 molecules (red) were seen in the outer-membrane (**I**) Membrane-bound or cytoplasmic HLA-DR molecules (red) were detected in HIV-1 provirus positive cells (**J**).

4. Notes

1. The base sequence corresponds to the region from 1379 to 1397 of the HIV-1 gag gene. As the melting temperature of the anti-parallel probe is higher than that of the parallel, the use of anti-parallel type is recommended. PNA probe can be dissolved with DEPC-treated water to a concentration of 100 µg/mL, aliquotted, and stored at –20°C. A 0.01% trifluoroacetic acid can be replaced instead of water.
2. Wear gloves throughout the steps until the hybridization step is completed. Use the dry-sterilized glassware equipment and those made by stainless steel (200°C, 2 h). Use autoclaved water (121ºC, 15 min) through the hybridization step.
3. To obtain an optimal concentration of proteinase K, treat fixed specimens with three different concentrations between 2 and 7 µg/mL of the enzyme.
4. This amplification method is based on the binding reaction of biotinylated tyramine to a phenol derivatives of a protein by peroxidase. This step sometimes gives nonspecific signals, therefore thorough pretreatment of specimens with methanol containing 0.3% H_2O_2 is essential to diminish the endogenous peroxidase activity. Also, it is important to stain two kinds of negative control to allow the identification of nonspecific signals: (1) HIV-1 DNA negative-specimen with a PNA probe and (2) HIV-1 DNA positive-specimen without a PNA probe (**Fig. 1**).
5. A single amplification method was successfully applied for HIV-1 RNA detection ***(16)***.
6. Considerable amounts of endogenous biotin is contained in liver, kidney, mucosa of digestive tract, and brain. Even in other organs, endogenous biotin becomes exposed through an activation step (*see* **Subheading 3.2.2., steps 2** and **4**). A biotin blocking of the endogenous biotin is recommended between the steps of stringent wash and HRP-FITC antibody reaction. Biotin blocking system (DakoCytomation A/S, X0590): (1) Incubate with avidin solution for 10 min, (2) wash in TBST three times for 3 min, (3) Incubate with biotin solution for 10 min, (4) wash in TBST three times for 3 min.
7. To measure the positivity of HIV-1 provirus in the CD4-positive T lymphocytes, we count 500 cells and calculate. The positivity of the HIV-1 provirus among 62 HIV-1-infected patients ranged between 0.3% and 7.9% (average of 2.7).

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Quantitative SNP-Detection Method for Estimating HIV-1 Replicative Fitness: Application to Protease Inhibitor-Resistant Viruses

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Abstract: **We have improved the methods for the standard competitive growth assay of human immunodeficiency virus type 1 (HIV-1). The cloning step for the mixed viral population and subsequent genotype analysis for arbitrary numbers of clones were excluded from procedures. Instead, a single nucleotide polymorphism (SNP)-detection step was devised for the determination of viral populations. The quantitative SNP-detection method can rapidly estimate the proportion of wild-type and mutant populations with high reproducibility. Consequently, this method allows manipulation of many samples within a short period. Using this new competitive growth assay, replicative fitness of drug-resistant HIV-1 containing an M46I amino acid mutation in the protease was assessed in the presence or absence of indinavir. Without indinavir, replicative fitness of wild-type HIV-1 surpassed that of M46I-mutated HIV-1, and the fraction of mutated virus was reduced to about 10% at passage #9. In contrast, the fraction of M46I-mutated virus increased to >90% at passage #5 in the presence of 26.4 nM indinavir. Almost identical results were obtained for L90M-mutated HIV-1 with or without saquinavir. HIV-1 can survive under indinavir pressure by acquiring M46I mutation, as with acquisition of the L90M mutation under saquinavir pressure. However, these mutations damage viral replicative fitness under natural conditions without any drugs. Subtle differences between wild-type and mutant viruses are thus easily detected using the improved method.**

Key words: **Quantitative SNP-detection, Competitive growth assay, Viral replicative fitness, Protease inhibitor-resistant HIV-1**

Competitive growth assay is a sensitive method for evaluating replicative fitness between 2 types of virus. In fact, this type of assay demonstrated the merit in determining minute differences in replicative fitness between viruses (1, 6). In this assay, viruses are dually inoculated into recipient cells and passed several times. Viral population at each passage is determined to judge which virus predominates. However, the procedure for standard competitive growth assay contains a cloning process for determining viral genotypes (19). This cloning process requires substantial time and labor, and arbitrary numbers of clones must be chosen from numerous clones for estimating viral populations. To bypass the cloning process, we established a quantitative single nucleotide polymorphism (SNP)-detection method for estimating viral populations in the competitive growth assay. This method allows differential determination of viral genotypes and estimation of proportions with high reproducibility. We evaluated the usefulness of this method by assessing the replicative fitness of protease inhibitor-resistant human immunodeficiency virus type 1 (HIV-1) possessing an M46I or L90M amino acid mutation (2, 9, 20, 22).

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Abbreviations: $CCID_{50}$, 50% cell culture infective dose; CPE, cytopathic effect; FCS, fetal calf serum; HIV-1, human immunodeficiency virus type 1; IC_{50}, 50% inhibitory concentration; K_m, Michaelis constant; MTT, methylthiazolyldiphenyl-tetrazolium bromide; PR, protease; RT-nested PCR, reverse transcription-nested polymerase chain reaction; SNP, single nucleotide polymorphism.

Materials and Methods

Cell culture. COS-7 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS) and 20 μg/ml gentamycin. MT-4 cells were maintained in RPMI1640 with 10% FCS and 20 μg/ml gentamycin. Cells were cultured at 37 C with 5% CO_2 in a humidified chamber. All materials for cell culture were obtained from Invitrogen (Tokyo).

Construction and expression of HIV-1 infectious clones. The infectious clone of pHXB2·PR_{M46I} was generated from the pSUM9-based plasmid (kindly provided by Dr. H. Mitsuya, National Cancer Institute, Bethesda, U.S.A.) by site-directed mutagenesis in which ATG at codon 46 of the protease gene was changed to ATA, ATT or ATC (21). As ATA is a common codon in PR inhibitor-resistant viruses possessing the M46I mutation, we used this type of infectious clone for analysis of viral replicative fitness. Other ATT- and ATC-type clones were used only in preparing template DNAs for kinetics analysis of single nucleotide labeling, as described below. Another infectious clone, pHXB2·PR_{L90M}, was made using the same method, but with TTG at codon 90 of the protease gene changed to ATG. Next, 400 ng of each infectious clone was transfected into COS-7 cells (about 40% confluence in 1 ml of medium) with lipofectamine (Invitrogen). After 4 days, 500 μl of culture supernatants were transferred to a fresh culture of MT-4 cells in a logarithmic phase for a cell density of 1×10^5 cells/ml in a total volume of 5 ml. After another 4 days, culture supernatants were harvested as virus stocks by spinning at $400\times g$ and stored at −80 C until use.

Inoculation of viruses into recipient cells. Recipient MT-4 cells were incubated with viruses at 37 C for 1.5 hr. After washing cells twice with phosphate-buffered saline, they were re-suspended in culture medium, and then cell culture was started. The detailed conditions of virus inoculation are described below.

Determination of viral titer. The 50% cell culture infective dose ($CCID_{50}$) of viral stocks was determined by the level of virus-induced cytopathic effect (CPE) (5, 17). The effect was triggered by infecting MT-4 cells (cell density, 1×10^5 cells/ml; total volume, 200 μl) with 5-fold serially diluted virus stocks in triplicate. The effect was assessed 5 days later using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) colorimetric method (16). Titers were estimated as 3.4×10^4 $CCID_{50}$/ml ($\pm3.9\times10^3$ $CCID_{50}$/ml) for HXB2, 3.3×10^3 $CCID_{50}$/ml ($\pm3.1\times10^2$ $CCID_{50}$/ml) for HXB2·PR_{M46I} and 3.2×10^3 $CCID_{50}$/ml ($\pm5.3\times10^2$ $CCID_{50}$/ml) for HXB2·PR_{L90M}.

Drug susceptibility assay. Serially diluted drug solutions (100 μl) were added to 100 μl of 2×10^4 MT-4 cell suspensions inoculated with 10 $CCID_{50}$ of each virus. Tests used 7 concentrations of indinavir and saquinavir, from 1,000 ng/ml to 0.064 ng/ml. After 5 days of culture, living cells were assessed using the MTT colorimetric method, and 50% inhibitory concentration (IC_{50}) of virus-induced CPE was then determined (16, 17). Each experiment was performed in triplicate. Indinavir and saquinavir were generously donated by Merck & Co. (Linden, U.S.A.) and F. Hoffmann-La Roche (Basel, Switzerland), respectively.

Competitive growth of viruses. First, 50 $CCID_{50}$ each of wild-type HXB2 and either HXB2·PR_{M46I} or HXB2·PR_{L90M} were inoculated into 2×10^4 MT-4 cells, then co-cultured in 200 μl of medium. Every 4 days, viruses in 10 μl of culture supernatant were passed into fresh MT-4 cell culture to allow virus growth in optimal conditions. Viral RNA at each time point was purified from total harvested culture supernatant using a High Pure RNA Isolation Kit (Roche, Tokyo). DNA fragments containing the protease gene of wild-type and mutated HIV-1 were amplified by reverse transcription-nested polymerase chain reaction (RT-nested PCR) and purified from the reaction mixture, as previously described (8). Finally, the population of each virus was differentially determined using a quantitative SNP-detection method developed for this study.

Single nucleotide labeling. Template DNAs (1,539 bp) used in generating standard curves and those used in a kinetics analysis were amplified by PCR with K4 and U12 primers (8), in which each plasmid of the infectious clone was individually added to a reaction mixture. Amplified DNAs were purified using a QIAquick gel extraction kit (Qiagen, Tokyo), and DNA concentrations were determined from triplicate measurements by a BioSpec-1600 spectrometer (Shimadzu, Kyoto, Japan). Single nucleotide labeling was performed using a SNaPshot Multiplex kit (Applied Biosystems, Tokyo). The Ready Reaction Mix contained 4 kinds of dideoxynucleotide labeled with different fluorescence dyes: ddGTP, dR110; ddATP, dR6G; ddTTP, dROX; and ddCTP, dTAMRA. Reaction mixture (total volume, 10 μl) contained 5 μl of Ready Reaction Mix, 20 nM of template DNAs, and 200 nM of SNP PR46 or SNP PR90 primer. Template DNAs of 4 different concentrations (5, 10, 20 or 40 nM) were added into reaction mixtures for kinetics analysis. For determining the third nucleotide of protease codon 46, we adopted an antisense primer (SNP PR46, 5'-$(A)_{10}$TCT TAC TTT GAT AAA ACC TCC AAT TCC CCC TAT-3') because a sense primer showed possible stem structure formation through the 3'-terminal region. A sense

primer (SNP PR90, 5'-(A)$_{15}$GAC CTA CAC CTG TCA ACA TAA TTG GAA GAA ATC TG-3') was used for determining the first nucleotide of protease codon 90. After labeling, 1 unit of calf intestinal alkaline phosphatase (New England BioLabs, Beverly, U.S.A.) was added to dephosphorylate unincorporated dideoxynucleotide substrates. Thermal controls for labeling and dephosphorylation were performed in accordance with the protocols described by the manufacturers. For electrophoresis, 1/600-diluted labeling mixture was applied to a Genetic Analyzer 310 (Applied Biosystems). Each area was estimated using GeneScan software (Applied Biosystems).

Growth kinetics analysis. For growth kinetics analysis, 2×10^5 MT-4 cells were inoculated with 10 or 100 $CCID_{50}$ of each virus. Cells were cultured in 2 ml of medium for 8 days, and aliquots of culture medium were harvested and spun down to obtain supernatants every 2 days. Amounts of p24 in supernatants were quantified using a Retro-Tek enzyme-linked immunosorbent assay kit (ZeptoMetrix, New York, U.S.A.).

Results

Development of Quantitative SNP-Detection Method

Determining the ratio of mixed genotype populations is easy using the SNP-detection method. However, as the SNaPshot reagent was developed for qualitative detection of SNP, products labeled with each dideoxynucleotides did not yield equal intensities. Fluorescence intensity of a unit amount of primer labeled with ddGTP, ddATP, or ddTTP was therefore measured and normalized to that of ddCTP-labeled primer to obtain intensity ratios. Kinetics analysis of single nucleotide labeling showed that each kind of labeled products increased in a Michaelis-Menten manner and nearly reached a plateau of 20 nM template DNA (Fig. 1). Michaelis constant K_m values of dideoxynucleotides ranged from 7.2 to 7.9 nM, indicating that affinities of the 4 types of dideoxynucleotide to DNA polymerase were similar. Ratio of fluorescence intensity at 20 nM of template DNA concentration was as follows: G:A:T:C =2.51 : 1.55 : 1.34 : 1.00. We subsequently generated a standard curve for determining the mix ratio of DNA fragments. Standard DNA mixtures with 6 different ratios of wild-type and mutant DNA (0:100, 20:80, 40:60, 60:40, 80:20 and 100:0) were subjected to single nucleotide labeling, and reaction mixtures were analyzed using the Genetic Analyzer. Two types of fluorescence-labeled primers were detected at similar time points, one labeled with ddCTP (wild-type) and the other with ddTTP (mutant) through the codon 46 polymorphism; or one labeled with ddTTP (wild-type) and the other with ddATP (mutant) through the codon 90 polymorphism. *Y*-axis values were calculated using the intensity ratio determined above. The standard curves thus generated are shown in Fig. 2. Standard deviations ranged from 0 to ±1.0 in intra-assays (n=5) and from 0 to ±1.9 in inter-assays (n=15), indicating high reproducibility for this method. The standard curves generated at four different concentrations of template DNA (5, 10, 20, and 40 nM) were similar in both cases of PR codons 46 and 90 (data not shown).

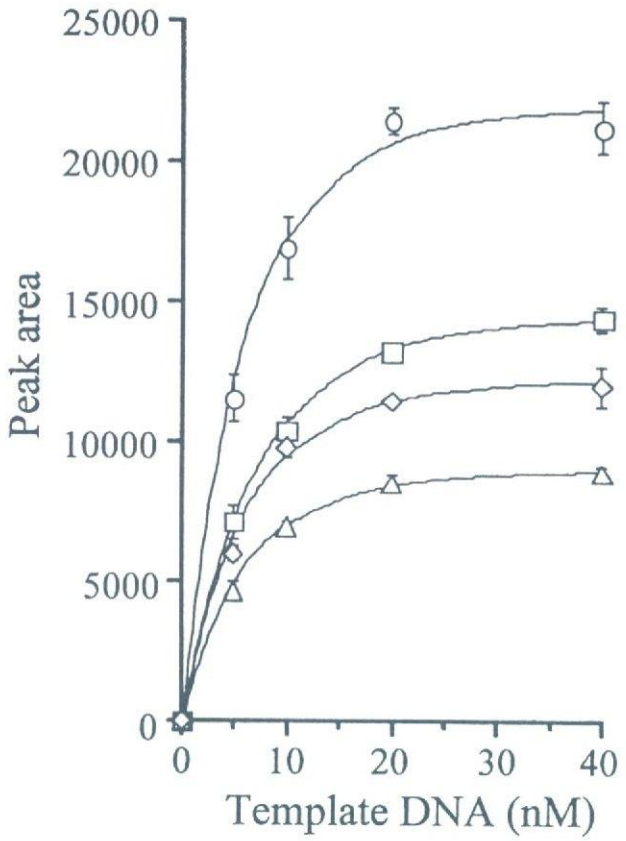

Fig. 1. Kinetics of single nucleotide labeling. SNP PR46 primers were individually labeled with different types of dideoxynucleotide, ddGTP (circles), ddATP (squares), ddTTP (diamonds), or ddCTP (triangles). Data represent means and standard deviations calculated from triplicate experiments.

Competitive Growth Assay of Wild-Type and Protease Inhibitor-Resistant HIV-1

Before starting the competitive growth assay, IC_{50} values for indinavir and saquinavir were determined. The IC_{50} of indinavir was 26.4±3.2 nM to HXB2 and 35.2±1.4 nM to HXB2·PR_{M46I}. Resistance of HXB2·PR_{M46I} compared to HXB2 was 1.3-fold. The IC_{50} of saquinavir was 8.1±1.2 nM to HXB2 and 17.4±2.7 nM to HXB2·PR_{L90M}. Resistance of

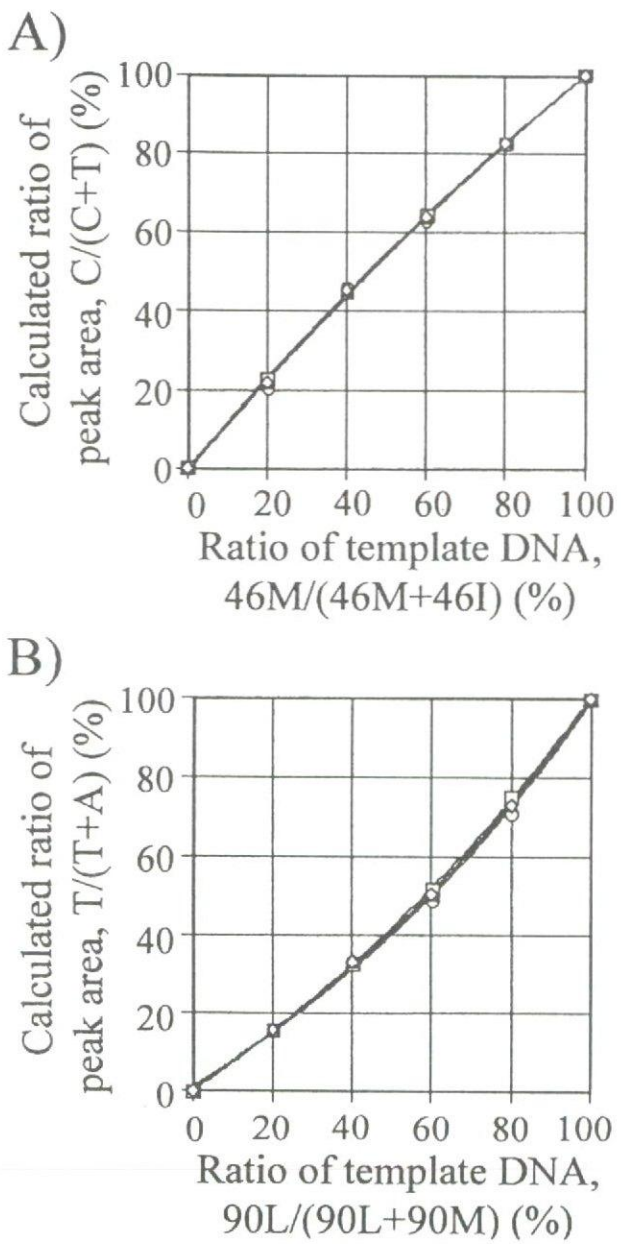

Fig. 2. Standard curves for estimating the ratio of viral populations in the co-culture of HIV-1. Standard curve for estimating the ratio of viral populations in the co-culture of HXB2 and HXB2·PR_{M46I} (A), or in the co-culture of HXB2 and HXB2·PR_{L90M} (B). Labeling reaction was performed with 20 nM template DNA. Three experiments were performed on different days, and 5 measurements were made. Day 1, squares; day 2, circles; day 3, triangles; and all data, diamonds. Standard deviations ranged from 0 to ±1.0 in intra-assays and from 0 to ±1.9 in inter-assays. Ratios of viral populations were estimated from calculated ratios of peak area using these standard curves.

HXB2·PR_{L90M} compared to HXB2 was 2.1-fold. Levels of drug resistance for HXB2·PR_{M46I} and HXB2·PR_{L90M} resembled previous descriptions (3, 10–12, 14, 15).

To evaluate the effects of M46I or L90M protease mutations on the replicative fitness of HIV-1, we directly compared replicative fitness of wild-type HXB2 with that of HXB2·PR_{M46I} or HXB2·PR_{L90M} using competitive growth assays. In co-culture containing 26.4 nM indinavir, the proportion of HXB2·PR_{M46I} increased from 51% at passage #1 to 75% at passage #3, and 95% at passage #7 (Fig. 3A, left). Conversely, the proportion of HXB2·PR_{M46I} decreased in a linear manner from 57% at passage #3 to 10% at passage #9 in the absence of indinavir (Fig. 3A, right). These results indicate that HIV-1 with the M46I mutation exhibits higher replicative fitness than wild-type HIV-1 in the presence of indinavir, but lower replicative fitness in the absence of this drug. Next, replicative fitness was compared between HXB2·PR_{L90M} and wild-type HXB2 in the same assay system. HXB2·PR_{L90M} completely dominated in co-cultures containing 8.1 nM saquinavir at passage #3 (Fig. 3B, left). In contrast, the proportion of HXB2·PR_{L90M} gradually decreased from 56% at passage #1 to 21% at passage #11 in the absence of saquinavir (Fig. 3B, right). These results indicate that acquisition of the L90M mutation is advantageous to HIV-1 replication in the presence of saquinavir, but reduces replicative fitness in the absence of the drug. To quantitatively evaluate relative replication efficiency of HIV-1 mutants, logarithm ratios against passage numbers of co-culture were plotted. Decreases in the proportion of both HXB2·PR_{M46I} and HXB2·PR_{L90M} were clearly apparent in the absence of any drug (Fig. 4), indicating that both M46I and L90M mutations damage the replicative fitness of HIV-1, although the level of damage is not serious. The nucleotide sequence of RNA genome was determined to eliminate the possibility that HIV-1 had captured any other mutations affecting viral replication during culture, but no such mutations were detected.

Growth Kinetics Analysis

Growth kinetics analysis is another common method for evaluating replicative fitness of HIV-1, although the sensitivity is lower than that of competitive growth assay (1, 19). We examined whether the reduced replicative fitness of HXB2·PR_{M46I} or HXB2·PR_{L90M} was detectable by growth kinetics analysis. After inoculation with 100 $CCID_{50}$ of HXB2, HXB2·PR_{M46I} and HXB2·PR_{L90M}, all viruses exhibited similar growth kinetics (Fig. 5A). Vigorous growth occurred to nearly 500 ng/ml of p24 for 4 days before reaching a plateau. Similar growth kinetics were also observed in the assay inoculated with 10 $CCID_{50}$ of viruses, 1/10 of the dose in Fig. 5A (Fig. 5B). No reduced replicative fitness of HXB2·PR_{M46I} or HXB2·PR_{L90M} was demonstrated by growth kinetics analysis. This result also implies that damage caused by the M46I and L90M mutations does