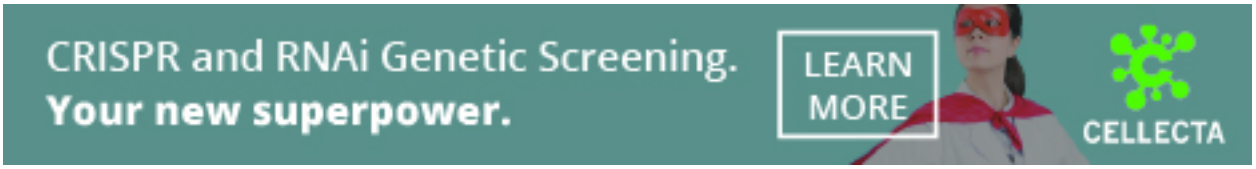
Rapid characterization of HIV-1 sequence diversity using denaturing gradient gel electrophoresis and direct automated DNA sequencing of PCR products.

B Andersson, J H Ying, D E Lewis, et al.

Genome Res. 1993 2: 293-300
Access the most recent version at doi:10.1101/gr.2.4.293



Rapid Characterization of HIV-1 Sequence Diversity Using Denaturing Gradient Gel Electrophoresis and Direct Automated DNA Sequencing of PCR Products

Björn Andersson, Ying Jia-Hsu, Dorothy E. Lewis,[1] and Richard A. Gibbs

Institute for Molecular Genetics and [1] Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

A direct method for visualization and isolation of sequence variants of human immunodeficiency virus type 1 (HIV-1) utilizing denaturing gradient gel electrophoresis (DGGE) combined with automated direct DNA sequencing was developed. Two fragments from the *env* gene and one from the *nef* gene of HIV-1, which together constitute approximately 1.0 kb of sequence, were amplified by PCR and analyzed. HIV-1 variants from each region were resolved and excised from the gel; this was followed by direct sequencing of different viral variants. In 9 infected patients, a limited number of dominant sequence variants could be seen in the three regions, together with a faint background of minor variants. The use of DGGE makes it possible to obtain a direct estimate of overall HIV-1 sequence diversity within patient samples without an intermediate DNA cloning step.

The nucleic acid sequence of the human immunodeficiency virus type 1 (HIV-1) varies greatly between and within individual isolates.[1] The extent of variation and the distribution of different HIV-1 forms has been extensively studied using PCR amplification followed by sequencing of individual subclones, to generate both a consensus sequence and representatives of minor subspecies.[2–8] These studies suggest that the distribution of HIV-1 sequence variants can be very broad, with many different forms represented in single isolates, and relatively small differences in abundance between the major and minor subspecies. Distributions have been found to range from a dominating form, constituting more than 50% of a sample together with several minor forms, to some cases where all of 20 sequences were found to be different.[4] Because of this extensive variation, it has been predicted that all HIV-1 genomes may be different, and even that there is no such thing as a single HIV-1 sequence.[5]

The method of sequencing cloned PCR products has the limitation, however, that only a small number of clones can be analyzed, and the statistical representation of the actual distribution is therefore poor. Balfe et al.[9] used an alternative method of limiting dilution of the patient DNA followed by PCR amplification to isolate different HIV-1 forms that were analyzed via direct DNA sequencing. In this way, the rate of evolution of HIV-1 was estimated by comparing HIV-1 sequences from a group of individuals infected from the same source. This method also results in a small number of sequences from each sample, which makes it difficult to draw conclusions about the distribution of different forms within an isolate.

Using direct DNA sequencing of undiluted PCR-amplified HIV-1 sequences, it is possible to characterize directly the predominant form(s) in patient samples. Because this method does not involve cloning, it yields an overall view of the distribution of different sequences.[10,11] This method has the disadvantage, however, that there is no physical separation of the different forms, thus making it impossible to determine the sequence of individual subspecies. By combining direct sequence analysis with a method to separate directly DNA molecules containing sequence differences, it would be possible to isolate the major forms and to obtain a direct estimate of the quantitative distribution of different sequences.

Several techniques to separate DNA fragments containing single-base differences have been described. These methods include single-strand conformation polymorphism (SSCP),[12] chemical mismatch cleavage,[13] and denaturing gradient gel electrophoresis (DGGE).[14] The method that has been found to be best suited for screening purposes and to have the broadest range of detectable changes in a sequence is DGGE with a GC-clamp.[15,16] Using DGGE, it is possible to separate DNA fragments differing by a single-base pair, due to their different melting properties.[14] DGGE has been used in many studies and it has

2:293–300©1993 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/93 $3.00

been shown that nearly all changes in a fragment result in separation by this method.

In the present study, the feasibility of using DGGE combined with direct sequence analysis for the determination of HIV-1 sequence variation with patient samples was investigated. DGGE was performed on PCR products from three different variable regions of HIV-1. The results show that it is possible to visualize directly the distribution of HIV-1 variants using DGGE, and that the individual variants can be isolated and their sequence determined by direct sequencing from DNA amplified from the isolated bands.

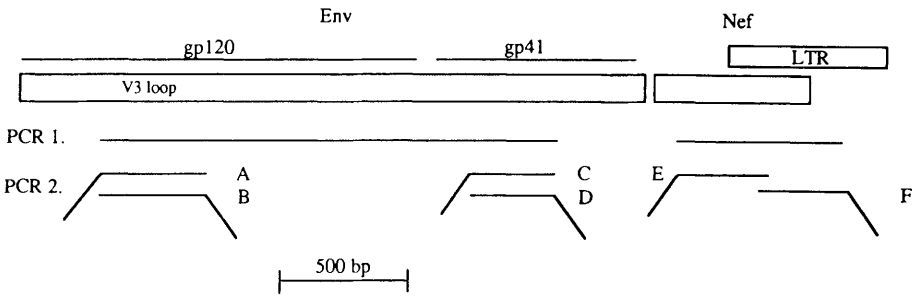


FIGURE 1 Schematic map of the distal portion of HIV-1, including the *env* and *nef* genes. To obtain DNA for DGGE analysis, PCR was performed in two steps. The approximate position of the different amplified fragments is shown beneath the map. In the first step (PCR 1.), one fragment was amplified from the *env* and *nef* genes, respectively. One-hundredth of this PCR was used in PCR 2. where fragments A–F were generated. The diagonal tails indicate the position of a 40-bp GC-clamp in each fragment.

MATERIALS AND METHODS

Source of HIV-1 Samples

PNL4 DNA and the H9 cell line were kindly supplied by Dr. L. Donehower. The patient samples were from a cohort of HIV-infected patients who had been followed for as long as 7 years. All patients were examined following informed consent. Of the 9 patients included in this study, 3 were symptomatic (JH, MBM, and TH) and 6 were asymptomatic (RK, TW, DP, DH, RL and LB). All but RK, DP, and RL were taking AZT or ddI. All except JH had CD4 numbers greater than 200/mm^3.

Cocultivation

Peripheral blood was fractionated using Ficoll gradients and 5–10 × 10^6 mononuclear cells were taken for DNA extraction. Co-cultures of patient lymphocytes with phytohemagglutinin (PHA)-stimulated recipient lymphocytes were performed using a 1:1 ratio of donor to recipient cells in the presence of 10 units/ml interleukin-2 (Amgen, Thousand Oaks, CA).

PCR

Nested PCR was used for the PCR amplification of three different regions of HIV-1 from genomic DNA prepared as described.[17] The primers used are listed in Table 1 and their corresponding positions in the HIV-1 genome can be seen in Figure 1. Primer sequences were chosen to be complementary to conserved regions of the virus. All PCR reactions were performed using a GeneAmp PCR system 9600 thermocycler (Perkin-Elmer Cetus). The first-step PCR reactions were performed in 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HC1 (pH 8.4), 200 μM of each dNTP, 25 pmoles of each primer, 100–300 ng of genomic DNA, and 2.5 units AmpliTaq DNA polymerase (Perkin-Elmer Cetus) in a total volume of 50 μl. PCR was performed as follows: 30 sec at 94°C, 30 sec at 60°C (with minimum ramp times) and after a ramp time of 30 sec, 72°C for 30 sec, repeated for 25 cycles. Second-step PCRs were performed with one internal primer using 0.5 μl from the first-step reaction as template. In the second-step reactions, one of the primers contained a 40-base GC-rich tail (GC-clamp), to form a high melting temperature domain in one end of the fragment. The GC-clamp sequence was as described by Sheffield et al.[15] Reaction conditions were as above except for the addition of 10% DMSO, 400 μM of each dNTP, 55°C annealing temperature, and a cycle number of 30. Oligonucleotides were synthesized using a 380B DNA synthesizer (Applied Biosystems). The GC-clamp primers were purified using HPLC.

Theoretical Melting Profiles

The theoretical melting profiles were ob-

TABLE 1 PCR Primers Used in This Study

Primer name	Position	Sequence 5′ → 3′
R185	A5′	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCAGCACAGTACAATGTACACATGG
4077	A3′	TTACAGTAGAAAAATTCCCCTCCACAA
1120	B5′	CAGCACAGTACAATGTACACATGG
R184	B3′	GCGGGCGGCGCGGGGCGCGGGCAGGGCGGCGGGGGCGGGCTTACAGTAGAAAAATTCCCCTCCACAA
R154	C5′	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTTGGAATGCTAGTTGGAG
1118	C3′	ATGGTGAATATCCCTGCCTAACTC
R183	D5′	CCTTGGAATGCTAGTTGGAG
R153	D3′	GCGGGCGGCGCGGGGCGCGGGCAGGGCGGCGGGGGCGGGCATGGTGAATATCCCTGCCTAACTC
R171	E5′	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCGCCACATACCTAGAAGA
R186	E3′	TCAGGGAAGTAGCCTTGTGTGTGG
Ro132	3′ of *nef*	TGCTAGAGATTTTCCACACTGAC

tained using the Melt-program described by Lerman and Silverstein[18] compiled for use via UNIX. The plots were made using x_grtool on UNIX. A 50% probability of melting at each position was used for the profiles shown (Fig. 2).

Denaturing Gradient Gel Electrophoresis

DGGE was performed using 7.5%, 19:1 acrylamide/bisacrylamide, polyacrylamide gels containing 1× TAE (40 mM Tris-acetate, 1 mM EDTA) buffer and a gradient of urea and formamide. Denaturant concentrations ranged from 0 (0%) to 7 M urea and 40% (vol/vol) formamide (100%). For the two *env* gene fragments, a gradient of 0–50% denaturant was used, and for the two *nef* gene fragments 0–75% denaturant was used. A gravitational gradient mixer (Hoefer Scientific Instruments) was used for casting the gradient gels. The gradients were either perpendicular or parallel to the direction of eletrophoresis. Electrophoresis was performed using a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories) that had been modified to allow the buffer in both the inner and outer buffer chamber to be in contact with the gel glass plates as described by Smith-Sørensen et al.[19] The electrophoresis cell was submerged in 1× TAE buffer heated to 59°C in a buffer tank supplied by Green Mountain Lab Supply, Inc. Electrophoresis times were between 1 hr, 15 min and 2 hr at 85 V. The gels were stained with ethidium bromide and the DNA was visualized using a UV transilluminator.

Isolation and DNA Sequence Analysis of Individual Forms

Individual bands were excised from parallel denaturing gradient gels. The gel slices were immersed in 40 μl of H_2O and frozen at −80°C for 1 hr, thawed at room temperature, frozen as before, and thawed at +4°C overnight. A small portion (0.25 μl) of the gel slice solution was used to reamplify the fragment using one primer that was biotinylated at the 5′ terminus and an opposing primer with a tail corresponding to the universal M13 sequencing primer recognition sequence. Single-stranded sequencing templates were prepared using magnetic beads (Dynal) and sequence analysis was performed using Sequenase (United States Biochemicals) and a DNA sequencer (Applied Biosystems). The sequencing procedure has been described in detail elsewhere.[17]

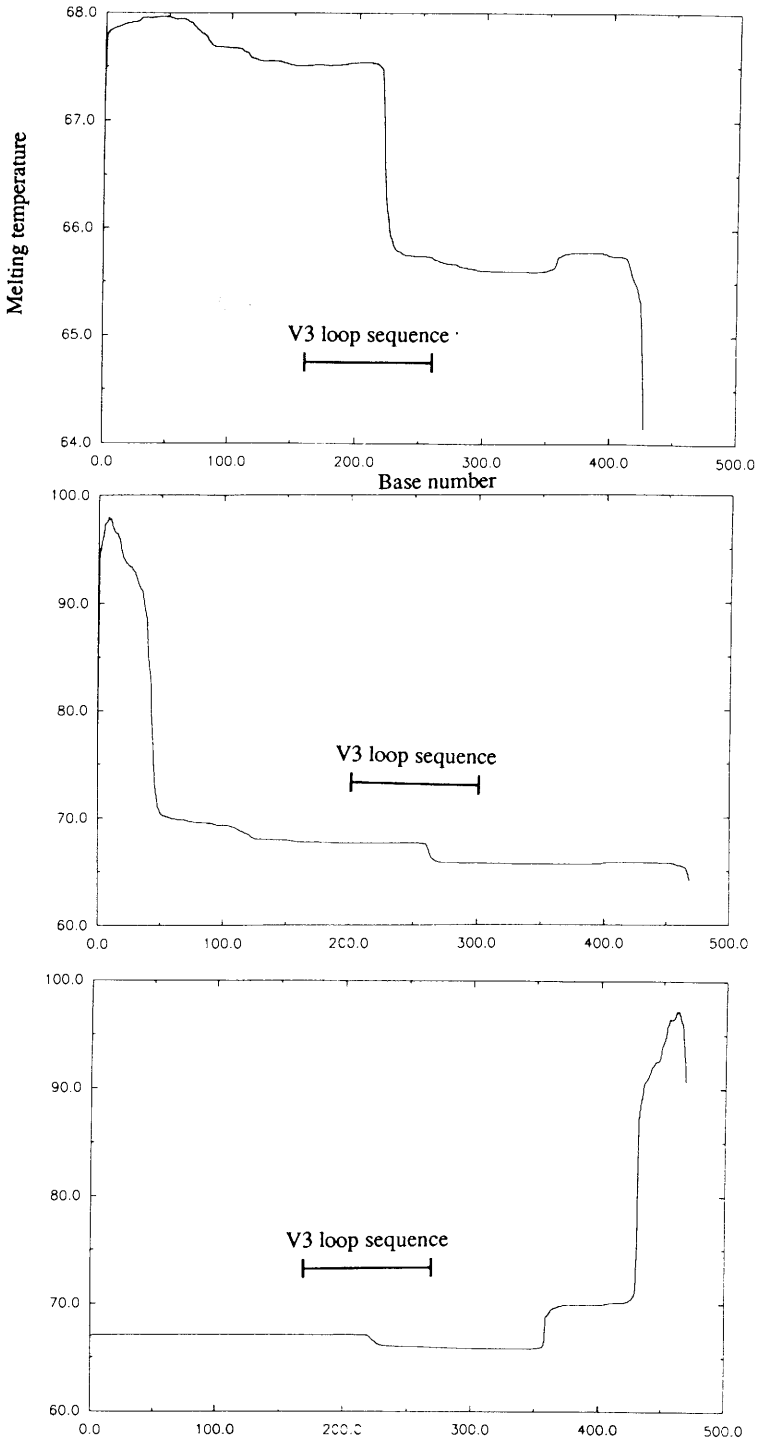


FIGURE 2 Theoretical melting profiles, showing the temperature where there is 50% probability of melting in each position for a 432-bp fragment from the HIV-1 *env* gene. The melting temperature is on the *y* axis while the fragment base number is on the *x* axis. Melting profiles for the unmodified fragment, (*top*) the same fragment but with a 40-bp GC-clamp in the 5′ end (fragment A, *middle*) and with a GC-clamp in the 3′ end (fragment B, *bottom*) are shown.

RESULTS

Regions Analyzed

DGGE analyses were performed on four different PCR-amplified fragments from

HIV-1 (Fig. 1). Primers 1120 and 4077 amplify a 432-bp fragment of the gp120 reading frame of the *env* gene. Included in this fragment is the sequence coding for the principal neutralization domain of HIV-1, the V3-loop. GC-clamp primers for each end of the fragment were synthesized (R185 and R184) to generate fragments A and B. A fragment spanning 319 bp of the gp41 reading frame of the *env* gene was amplified using primers R183 and 1118 and corresponding GC-clamp primers were synthesized (R154 and R153, which were used to amplify fragments C and D). A 420-bp fragment amplified using primers R171 (GC-clamp primer) and R186 covers the 5′-most part of the *nef* gene. This fragment (E) has a GC-clamp in the 5′ end. All fragment lengths are given for the HIV-1 reference strain HXB2. The exact lengths vary between different isolates.

Melting Properties of the Fragments

Theoretical melting profiles for the different fragments from the reference sequence HXB2 and for the predominant sequence from the patients described in Burger *et al.*[11] were obtained to predict which fragments were suitable for DGGE analysis. The overall pattern of melting domains was similar for each fragment in the different HIV-1 genomes, despite some sequence differences. Theoretical melting profiles for the fragment containing the V3 loop sequence, unmodified and with the GC-clamp attached to either end (fragments A and B) for the HXB2 reference genome, are shown in Figure 2. In the absence of a GC-clamp, the fragment shows three major melting domains at approximately 68, 67.5, and 66°C respectively, with the highest melting domain at the 5° end and the lowest at the 3° end. This results in a fragment A melting profile in which the same three melting domains can be seen, together with the high melting domain formed by the GC-clamp sequence, and where the lowest melting domain is located in the 3° end of the fragment. In contrast, fragment B has a melting profile with a long 67°C melting domain in the 5′ end of the fragment followed by a 65°C domain, a 70°C domain, and the GC-clamp high-melting domain. This pattern was expected possibly to cause irregular melting of fragment B due to the position of the lowest melting domain with the fragment, which would make it unsuitable for DGGE analysis. On the other hand, fragment A was expected to be suitable for analysis. In a similar fashion, fragment C was expected to be more suitable for DGGE than fragment D, and Fragment E was expected to be suitable for DGGE analysis.

The theoretical predictions were each confirmed using perpendicular DGGE of all fragments. Fragments A, C, and E all showed clear stable melting curves (fragment C from H9 and from patients RK and TH are shown in Fig. 3). Perpendicular DGGE of fragments B and D resulted in unclear, interrupted melting of some domains (data not shown), which may cause problems in mutation detection. Subsequently, fragments A, C, and E were used for further analysis. It is likely, from the theoretical melting curves and from the perpendicular DGGE, that it is possible to detect differences in a large part of the sequence of each fragment. The fragments used in this study are relatively long (319–434). Thus, in the higher melting domains closest to the GC-clamp that melt relatively late during DGGE, some changes may be undetectable because their effect on the migration of the fragments will be small. This is also indicated by theoretical calculations using the program *sqhtx*. To detect these differences, narrow gradients spanning a high denaturing range are necessary. The exact conditions for these may be difficult to determine, however, due to the may sequence differences between different strains of HIV. Therefore, broad gradients have been used in this study, which means that mutations in some melting domains are probably not as clearly separated as changes in the other domains.

DGGE of Control Samples

DGGE of fragments A, C, and E was performed on amplified samples from cloned HIV-1 genome (PNL4, courtesy of L. Donehower), which was expected to contain only one HIV-1 sequence. The analysis revealed a single dominant band on DGGE for all fragments after 30

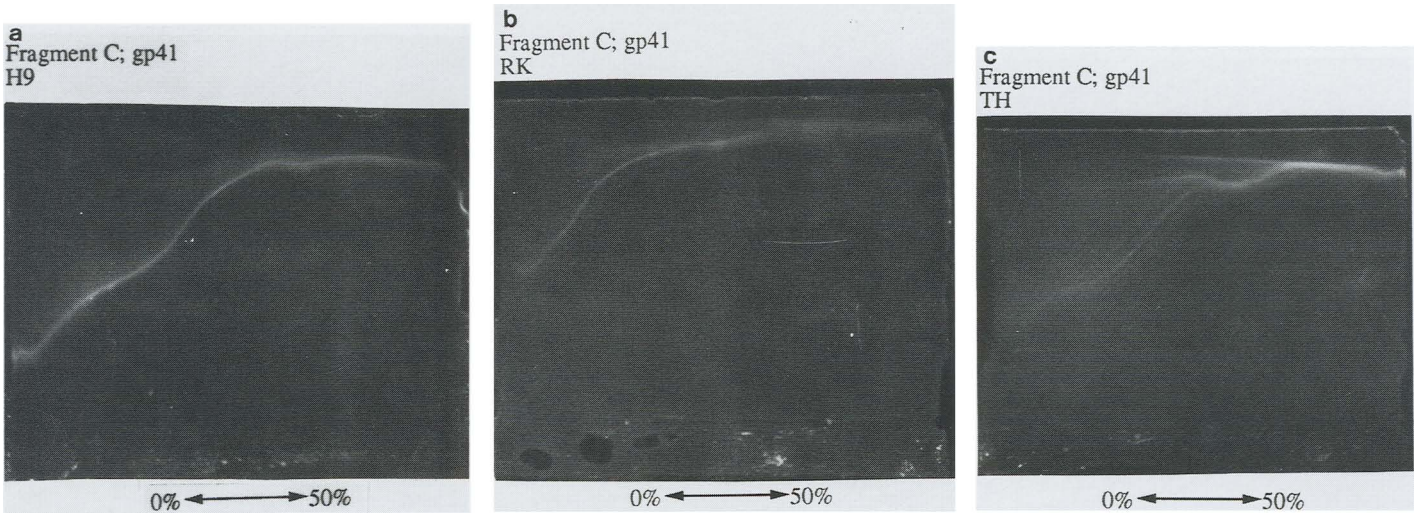


FIGURE 3 Perpendicular DGGE of amplified fragment C from cell line H9 (*a*), and from patients RK (*b*) and TH (*c*). Electrophoresis was performed at 60°C and 85 V for 2 hr, and the denaturing gradient range was 0–50%. For H9 and RK, only one dominating form can be detected together with one minor form in RK, whereas for TW four major homoduplex bands together with faint heteroduplexes can be seen.

cycles of PCR (Fig. 4). For all fragments, a faint background smear with possible weak bands present could be observed (Fig. 4), and for fragment A two clear minor bands could be seen (Fig 4a,b). This background may be due to *Taq* polymerase errors during PCR, but it is also possible that minor sequence variants were present in the plasmid preparation. DGGE was also performed on fragments amplified from DNA from the HIV-1-infected cell line H9, which resulted in a pattern similar to that of PNL4 in that one major form could be detected. Due to the extensive sequence variation between different strains of HIV-1, the major variants of fragments A and E from H9 and PNL4 showed different migration on DGGE, while for fragment C, no difference between the two strains could be detected.

DGGE of Patient Samples

The heterogeneity of amplified HIV-1 DNA samples was surveyed in a panel of infected patients. All DNA samples were obtained directly from patient blood samples without cocultivation. All patient samples from each region migrated differently from each other on parallel denaturing gradient gels, which confirms the extensive sequence variation among patient isolates of HIV-1. In general, the DGGE profiles could be divided into two categories; one where one quantitatively dominant form could be separated from some minor ones, and a second group where several dominant forms could be seen.

Samples from 9 patients were analyzed for diversity in the two *env* fragments, A and C, and 8 patients were analyzed for the *nef* gene. For fragment A, 2 patients showed one major form (TH and LB) while four patients (TW, MBM, JH, and RL) showed patterns with two to four dominant variants (Fig. 4a,b). Fragments from three of the patients (RK, DH, and DP) were not interpreted because of low amounts of DNA on the gel. Similar results were obtained for fragment C, where 5 of the patients (RK, TW, DH, RL, and DP) had one dominant form and 4 patients showed more variable patterns (MBM, TH, JH, and LB; Fig. 4c,d). The fastest migrating bands in patients DH and LB were shown to be nonspecific PCR products by using perpendicular DGGE, where it could be seen that the melting pattern of these fragments is completely different from that of fragment C, indicating that the nonspecific fragment lacks a GC-clamp (data not shown).

For fragment E from patient TW, only one dominating form could be detected, with small amounts of a small number of minor forms. Amplified samples from the remaining patients showed more varied patterns with three to five forms present in equal amounts (Fig. 4e). Sample RL was not interpreted because of the low amount of PCR product. For all three regions, perpendicular DGGE in the relevant melting intervals was performed for approximately half the samples analyzed and the same patterns of variants as in the parallel gels were obtained.

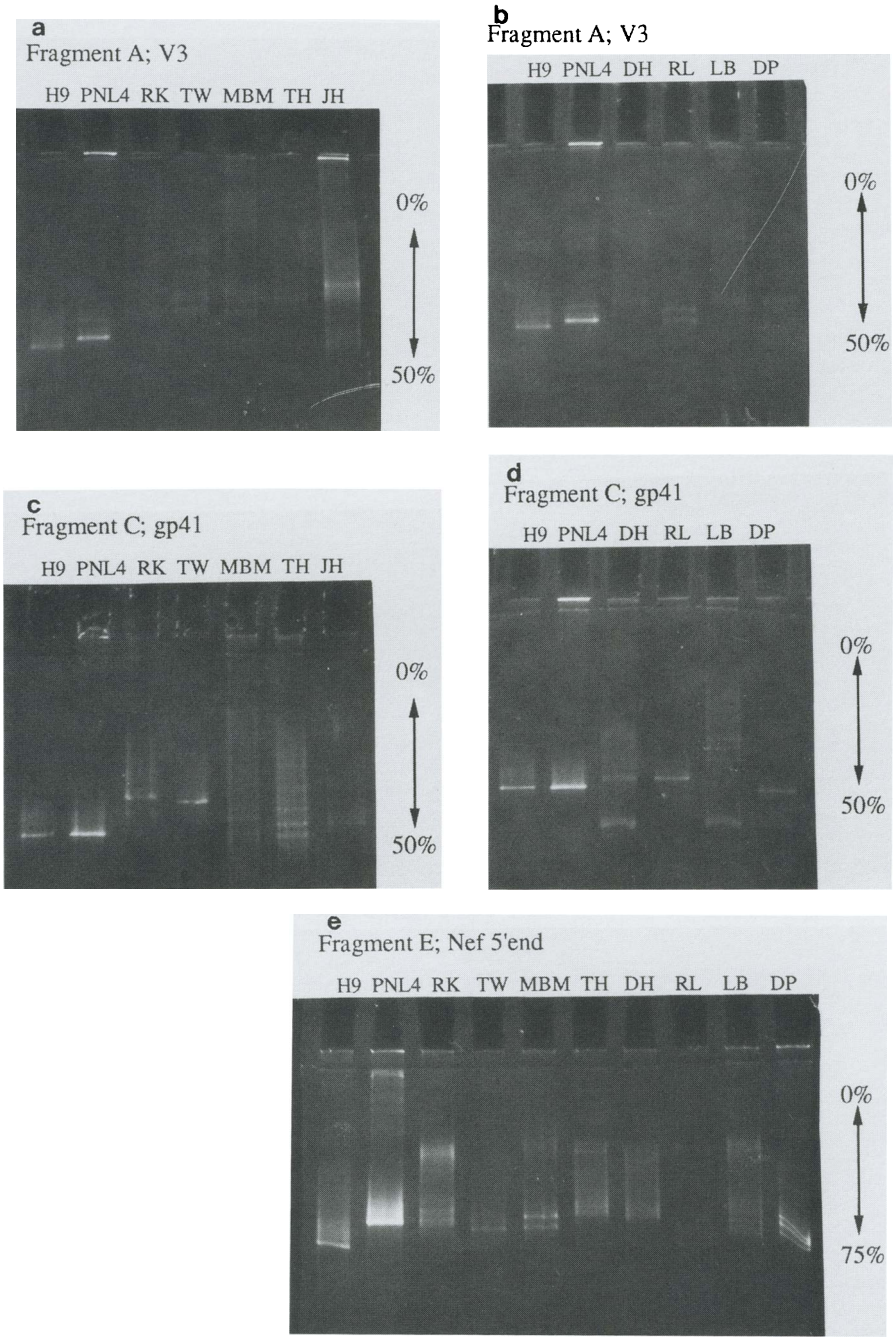


FIGURE 4 Parallel DGGE of amplified HIV-1 sequences from patient samples. The gradient gels were electrophoresed at 60°C and 85V for 2 hr. For fragments A and C, a gradient ranging from 0 to 50% was used, while 0–75% was used for fragment E. Gels in *a* and *b* show DGGE of fragment A, fels *c* and *d* show for fragment C, and the gel in *e* shows fragment E. For fragment A, patients TH and LB, for fragment C, patients RK, TW, DH, RL, and DP, and for fragment E, patient TW showed a pattern where a single dominant variants could be detected.

Often, when there was a relatively high yield of PCR product, several forms melting at low denaturant concentrations could be detected. This can be seen for sample TH, fragment E, for example (Fig. 4e). These bands are characteristic of heteroduplexes formed in the later cycles of PCR. In addition, a background smear was often present, which was probably due to various sequence changes introduced by *Taq* polymerase during PCR (see above).

The results of the patient survey show that it is possible to separate different forms for all fragments tested, and that it is possible to resolve dominant and minor variants in single PCR-amplified patient samples. The dominant forms within each sample are limited to one or a few, which can be analyzed further (see below).

Effects of Cocultivation

Peripheral blood mononuclear cells (PBMC) from a patient infected with HIV-1 (patient JH) were cocultivated with PHA-activated PBMCs from an uninfected donor. Samples were taken at 11 and 15 days after initiation of culture, and DGGE analysis was performed on amplified fragments A and C (V3 and gp41) from both time points and from DNA prepared directly from a blood sample from the patient. The results (Fig. 5) were similar for both fragments, and show that the pattern of variants changes during culture, so that a different major variant dominates after culture (see arrows in Fig. 5). This result is in accordance with results obtained through cloning and sequencing PCR products.[2,4,5]

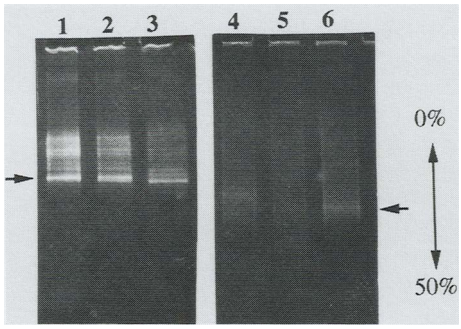


FIGURE 5 Parallel DGGE of fragments A and C from patient JH, amplified either directly from a patient blood sample or after cocultivation of patient cells with peripheral blood mononuclear cells from a healthy donor. DGGE was performed at 60°C and 85V for 2 hr, and the gradient range was 0–50%. (Lanes *1–3*) Fragment C amplified, after 15 days of cocultivation, after 11 days, and directly, respectively; (lanes *4–6*) fragment A amplified directly, after 11 days of cocultivation, and after 15 days, respectively. The arrows indicate the dominant variants after cocultivation.

Isolation and Sequencing of Different Forms

The dominant bands observed after DGGE of fragment C from patient TH (Fig. 3a) were excised from a parallel gradient gel and reamplified using the same primers as in the original amplification (R154-1118). When the reamplified sample was analyzed using DGGE, a strong enrichment for the desired band could be seen (the desired band constituting >90% of the total product; data not shown). Reamplification with primers for sequence analysis was performed as described in Materials and Methods, and high-quality sequence information was generated. Clear single-base differences between the different forms could be detected. For example, the difference between the two fastest migrating forms in fragment C from fragment TH was found to be a G:C–A:T difference (Fig. 6) located approximately 70 bases from the 3′ end of the fragment. The difference is located in the lowest melting domain and is therefore expected to be easily detected by DGGE. The form containing the AT base pair melts at a lower denaturant concentration, and thus migrates a shorter distance in the parallel DGGE.

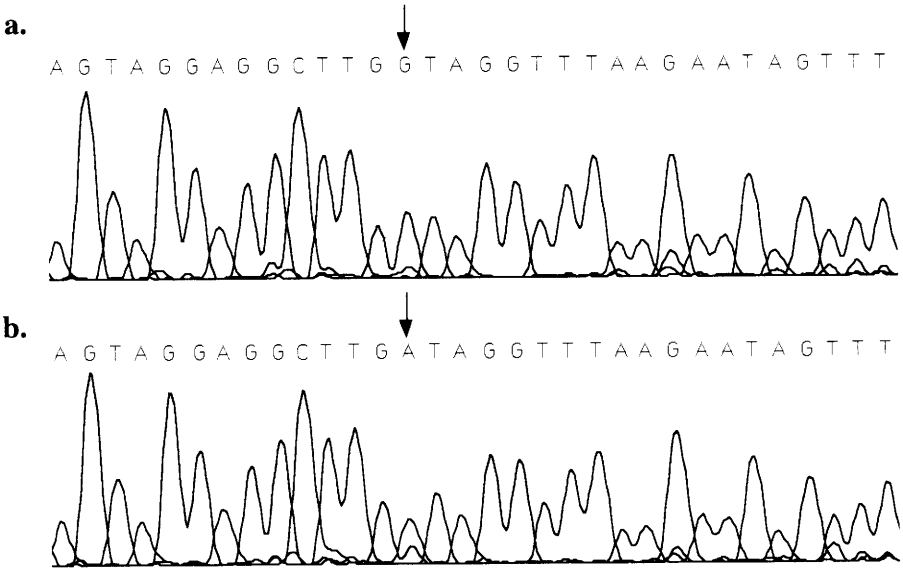


FIGURE 6 Automated direct sequence analysis of variants of fragment C from patient TH. The different forms were separated by parallel DGGE (see Fig. 3c), excised from the gel, extracted by repeated freezing, and reamplified for sequencing. The two forms show a single base difference, which is indicated by arrows. Form *a* contains a G:C base pair at the varying position and therefore melted at a higher denaturant concentration.

DISCUSSION

DGGE has been demonstrated here to be a useful tool for the study of populations of HIV-1 DNA fragments amplified directly from patient samples. Theoretical calculations and results from using this method in several different systems indicate that practically all sequence changes in a lower melting domain can be detected.[20–22,15] When multiple sequence variants are present, the analysis is facilitated by the presence of only one band in the gel per unique sequence. The DNA fragments are not modified during the assay and the gels are easy to handle, so that individual variants can be isolated and reamplified for subsequent sequence analysis. Together, these features make it possible to separate different HIV-1 variants, and ultimately to determine their precise base differences.

Three different regions of HIV-1, spanning a total of approximately 1100

bases, have been analyzed here. The calculated melting properties suggest that the current GC-clamped fragments and DGGE conditions will allow detection of sequence changes in at least two-thirds of these fragments. The remaining bases are in higher melting domains close to the GC-clamps and may require DGGE with different conditions for reliable mutation detection. The exact amount of sequence available to DGGE analysis in these fragments will become more clear as additional fragments are isolated and sequenced.

Both perpendicular DGGE and parallel DGGE have been performed. Perpendicular gradient gels have the advantage that non specific PCR products, when present, do not confuse the analysis to the same extent as in parallel gels. However, the resolution is superior in the parallel gradient gels, where the bands are concentrated when the migration is retarded. Routinely, perpendicular gels are performed for a few samples to determine the overall melting properties of the region, and when it is needed to clarify the pattern of variants.

Using DGGE, it was possible to separate HIV-1 variants in all regions tested. Variation both between and within patient samples could be detected, and both major and minor forms could be identified. It was confirmed that when lymphocytes from an HIV-1-infected patient are concultivated with peripheral blood mononuclear cells (PBMCs) from a healthy donor, a minor variant in the patient sample can become the dominant variant after cocultivation.

The limit of detection of HIV-1 variants that are present in low amounts is probably determined by the background caused by *Taq* DNA polymerase errors. It may be possible to increase the detection sensitivity by using Vent DNA polymerase, which has proofreading activity and therefore a lower error rate.[23] It is also possible that minor variants can be obscured through heteroduplex formation with the major variants in the later PCR cycles. This can be avoided by limiting the number of PCR cycles. No more than 25 and 30 cycles were used for the first- and second-step PCR reactions, respectively, to minimize changes in the distribution of variants arising due to saturation of the PCR reaction. The possibility that the pattern of different forms could be affected by *Taq* polymerase errors occurring during PCR was tested by repeated runs from samples with multiple forms. The patterns of sequence variants were virtually indistinguishable when samples were repeated in this way (data not shown).

DGGE is expected to resolve almost all variants in the suitable melting domains, and because there is no intermediate cloning step the whole population of products is analyzed. Therefore, DGGE gives a direct representation of the distribution of different sequence variants in the amplified samples. Overall, the diversity of different HIV-1 variants within the patient samples analyzed here by DGGE appears different from that previously described using the method of cloning and sequencing of PCR products. DGGE suggests that the major variant(s) may be quantitatively dominant to a greater extent than what has been estimated using sequence analysis of cloned PCR products. The overall distribution of HIV-1 variants within different patient samples will be investigated.

Direct sequence analysis of DNA reamplified from single bands excised from denaturing gradient gels showed that it is possible to characterize different HIV-1 sequence variants unambiguously using this approach, despite the presence of multiple variants. Direct sequence analysis was performed using magnetic beads for isolation of single-stranded templates combined with a universal priming site introduced via PCR and fluorescent sequence analysis. Using this method high-quality sequence information could be reliably obtained (Fig. 6).

In conclusion, a technique using DGGE and direct automated sequence analysis for direct characterization of the distribution of sequence variants with HIV isolates is presented. Variants can be separated in PCR-amplified fragments from different regions of HIV-1, and individual variants can be isolated from the gel and their sequence determined by direct automated sequence analysis. Using this method, variation within individual patient samples was detected and it was confirmed that cocultivation in PBMCs leads to changes in HIV-1 populations. Using DGGE, it will be possible to study changes in HIV-1 populations during different stages of the infection, after transmission of HIV-1 between individuals, in different cell types, and in proviral and free viral forms.

ACKNOWLEDGMENTS

The careful technical assistance of Derek Ng Tang, with the cocultivation, is gratefully acknowledged. We thank Prof. L.S. Lerman for access to the software used for theoretical melting calculations. This work was supported by the W.M. Keck Foundation and National Institutes of Health grant 5U01AI 30243-03 and Genome Program Center grant 21-272110105218.

REFERENCES

1. Myers, G., J.A. Berzofsky, B. Korber, and R.F. Smith. 1991. *Human retroviruses and AIDS 1991*. Los Alamos National Laboratory, Los Alamos, New Mexico.
2. Meyerhans, A., R. Cheynier, J. Albert, M. Seth, S. Kwok, J. Sninsky, L. Morfeldt-Månson, B. Åsjö, and S. Wain-Hobson. 1989. Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. *Cell* **58:** 901–910.
3. Wolfs, T.F., J-J. deJong, H. van den Berg, J.M.G.H. Tunagel, W.J.A. Krone, and J. Goudsmit. 1990. Evolution of sequences encoding the principal neutralization epitope of human immunodeficiency virus 1 is host dependent, rapid, and continous. *Proc. Natl. Acad. Sci.* **87:** 9938–9942.
4. Vartanian, J-P., A. Meyerhans, B. Åsjö, and S. Wain-Hobson. 1991. Selection, recombination, and G-A hypermutation of human immunodeficiency virus type 1 genomes. *J. Virol.* **65:** 1779–1788.
5. Delassus, S., R. Cheynier, and S. Wain-Hobson. 1991. Evolution of human immunodeficiency virus type 1 nef and long terminal repeat sequences over 4 years in vivo and in vitro. *J. Virol.* **65:** 225–231.
6. Holmes, E.C., L-Q. Zhang, P. Simmonds, C.A. Ludlam, and A.J. Leigh Brown. 1992. Convergent and divergent evolution in the surface envelope glycoprotein of human immunodeficiency virus type 1 within a single infected patient. *Proc. Natl. Acad. Sci.* **89:** 4835–4839.
7. Wolinsky, S.M., C.M. Wike, B.T.M. Korber, C. Hutto, W.P. Parks, L.L. Rosenblum, K.J. Kunstman, M.R. Furtado, and J.L. Munoz. 1992. Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. *Science* **255:** 1134–1137.
8. Ou, C-Y., C.A. Ciesielsky, G. Myers, C.I. Bandea, C-C. Luo, B.T.M. Korber, J.I. Mullins, G. Schochetman, R.L. Berkelman, A.N. Economou, J.J. Witte, L.J. Furman, G.A. Satten, K.A. MacInnes, J.W. Curran, H.W. Jaffe, Laboratory Investigation group, and Epidemiologic Investigation group. 1992. Molecular epidemiology of HIV transmission in a dental practice. *Science* **256:** 1165–1171.

9. Balfe, P., P. Simmonds, C.A. Ludlam, J.O. Bishop, and A.J. Leigh Brown. 1990. Concurrent evolution of human immunodeficiency virus type 1 in patients infected from the same source: rate of sequence change and low frequency of inactivating mutations. *J. Virol.* **64:** 6221–6233.
10. Wahlberg, J., J. Albert, J. Lundeberg, A. con Gegerfelt, K. Broliden, G. Utter, E-M. Fenyö, and M. Uhlén. 1991. Analysis of the V3 loop in neutralization-resistant human immunodeficiency virus type 1 variants by direct solid-phase DNA sequencing. *AIDS Res. Hum. Retrovir.* **7:** 983–989.
11. Burger, H., B. Weiser, K. Flaherty, J. Gulla, P-N. Nguyen, and R.A. Gibbs. 1991. Evolution of human immunodeficiency virus type 1 nucleotide sequence diversity among close contacts. *Proc. Natl. Acad. Sci.* **88:** 11236–11240.
12. Orita, M., Y. Suzuki, T. Sekiya, and K. Hayashi. 1989. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* **5:** 874–879.
13. Cotton, R.G.H., N.R. Rodrigues, and R.D. Campbell. 1988. Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. *Proc. Natl. Acad. Sci.* **85:** 4397–4401.
14. Fisher, S. and L.S. Lerman. 1983. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: Correspondence with melting theory. *Proc. Natl. Acad. Sci.* **80:** 1579–1583.
15. Sheffield, V.C., D.R. Cox, L.S. Lerman, and R.M. Myers. 1989. Attachment of a 40-base pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc. Natl. Acad. Sci* **86:** 232–236.
16. Theophilus, B.D.M., T. Latham, G.A. Grabowski, and F.I. Smith. 1989. Comparison of RNase A, a chemical cleavage and GC-clamped denaturing gradient gel electrophoresis for the detection of mutations in exon 9 of the human β-glucosidase gene. *Nucleic Acids Res.* **17:** 7707–7722.
17. Gibbs, R.A., P-N. Nguyen, A. Edwards, A.B. Civitello, and C.T. Caskey. 1990. Multiplex DNA deletion detection and exon sequencing of the hypoxanthine phosphoribosyl transferase gene in Lesch-Nyhan families. *Genomics* **7:** 235–244.
18. Lerman, L.S. and K. Silverstein. 1987. Computational simulation of DNA melting and its application to denaturing gradient gel electrophoresis. *Methods Enzymol.* **155:** 482–501.
19. Smith-Sørensen, B., E. Hovig, B. Andersson, and A-L. Bodørresen. 1992. Screening for mutations in human hprt cDNA using the polymerase chain reaction (PCR) in combination with constant denaturant gel electrophoresis (CDGE). *Mutat. Res.* (in press).
20. Cariello, N.F., P. Keohavong, A.G. Kat, and W.G. Thilly. 1990. Molecular analysis of complex cell populations: Mutational spectra of MNNG and ICR-191. *Mutat. Res.* **231:**165–176.
21. Myers, R.M., S.G. Fischer, T. Maniatis, and L.S. Lerman. 1985. Modification of the metling properties of duplex DNA by attachment of a GC-rich DNA sequence as determined by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* **13:** 3111–3129.
22. Myers, R.M., S.G. Fischer, L.S. Lerman, and T. Maniatis. 1985. Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* **13:** 3131–3145.
23. Ling, L.L., P. Keohavong, C. Dias, and W.G. Thilly. 1991. Optimization of the polymerase chain reaction with regard to fidelity: Modified T7, Taq, and Vent DNA polymerases. *PCR Methods Applic.* **1:** 63–69.

Received November 25, 1992; accepted in revised form February 1, 1993.