NORTHERN ILLINOIS UNIVERSITY

Deletion Mutants May Be the Key to Determining the Structure of the Vif Protein of HIV

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ABSTRACT

The Vif (viral infectivity factor) protein of HIV-1 enhances viral infectivity during virus production 100 to 1000 fold (4). Though Vif is required for HIV-1 infection in peripheral blood T lymphocytes, macrophages, and some T cell lines, its exact role during HIV-1 infection remains unknown (13). Currently, it is proposed that Vif may affect the late phases of the viral life cycle, such as virion assembly and maturation (4). In order to get a better understanding of the function and mechanism of Vif, obtaining the structure of the protein is of extreme importance. Unfortunately, no structural information is available. Determining the structure of a protein using NMR (nuclear magnetic resonance) spectroscopy requires that the protein be monomeric in solution. However, Vif exists in solution as aggregates, making NMR impossible. Constructing deletion mutants of Vif may change the protein's aggregation behavior to monomeric in solution. Thus, these mutants may be the key to determining the structure of Vif, which, in turn, may lead to a better understanding of its function and indicate possible HIV-1drug targets. PCR (polymerase chain reaction) was used to make the Vif deletion mutants. Restriction digests were performed on both the vector (pD10Vif) and the PCR product using the enzymes BamH1 and HindIII. A ligation and transformation were performed, and transformants were selected for using Ampicillin resistance. A plasmid prep was performed and another restriction digest was performed and run on an agarose gel to ensure the presence of the insert. The Vif mutant was then sequenced as an extra precaution before performing a protein expression test to determine whether or not the protein was soluble.

INTRODUCTION

Background

HIV has a spherical shape, with a diameter of 1/10,000-mm (10). Its outer coat is called the viral envelope, and is made of a lipid bilayer. Protruding from the envelope surface is the Env protein, with a cap made from 3 or 4 gp120 molecules and a stem made from 3 or 4 gp41 molecules. Made of 2000 copies of the protein p24, the capsid surrounds two single strands of HIV RNA within the envelope (10).

Like any retrovirus, HIV contains the genes Gag, Pol, and Env. The Gag gene is translated into the molecules that make up the capsid protein, while the Env gene is translated into the molecules of the

envelope protein. The Pol gene is transcribed into the molecules of reverse transcriptase, protease, and integrase (7). However, early into the infective process, Gag, Pol and Env are not made. Instead, RNA is first processed, creating six auxiliary proteins, each contributing to some aspect of the HIV-1 life cycle, which is briefly explained below.

> CD-4 Receptors on the host cell bind to the viral envelope protein, the envelope fuses with the cell membrane, and the viral capsid is taken into the cell. When the capsid opens, the viral RNA, reverse transcriptase, and tRNA primers are released into the host cell. A double-stranded DNA copy of the RNA is synthesized by the reverse transcriptase, and the DNA copy is integrated into the host cell genome, where it is then called a provirus. This provirus DNA is then transcribed into RNA to produce viral proteins, and the new virus particles are assembled and released from the cell by budding off (9).

The first of these auxiliary proteins is Tat (transcriptional activation protein). Tat is responsible for activating the transcription of the HIV-1 LTR (long-terminal repeat) promoter, and it is also necessary for viral replication (3). The Rev protein is responsible for the nuclear export of late HIV-1 mRNA (3). Nef (numerous effector functions) was once thought to be a negative elongation factor, but instead plays a role in the down-regulation of cell surface CD-4 and MHC-1. As a result, complexes formed between CD-4 and the HIV-1 envelope would be reduced, and cytotoxic T lymphocyte mediated lysis of infected cells would be inhibited (3). Nef is also thought to enhance virion activity. The protein Vpr mediates the import of HIV-1 preintegration complexes into the nucleus of non-dividing cells. It also arrests the cell in the G2 phase of the cell cycle, so that mitosis cannot occur (3). Unique to HIV-1, Vpu enhances the release of virions from infected cells and the selective degradation of CD-4 in a cell's endoplasmic reticulum (3). The least understood of these auxiliary proteins is Vif. A singly spliced mRNA that is dependent on Rev encodes this 192 amino acid protein (3).

Significance of Vif

Many ideas as to the role of Vif have been put forth, and will be discussed further on. Knowing the structure of Vif may tell a lot about its function and mechanism, and may lead to new and more successful drug therapies.

One of the reasons that the role of the 23 kDa Vif protein is so poorly understood is that only certain cells require Vif for efficient HIV-1 replication (13). The cells that require Vif for replication are termed nonpermissive, while cells that do not require Vif for replication are termed permissive (12). The fact that some cells require Vif and others do not suggests that Vif can somehow compensate for cellular

factors needed for production of infectious virus particles in some cell lines (permissive) but are absent in others (nonpermissive) (4).

Previous studies show that Vif deficient (Vif$^-$) mutants recovered from nonpermissive cells have abnormal features, such as reduced quantities of envelope protein and altered quantities of the Gag and Pol proteins (1). Furthermore, a Vif$^-$ virus produced in a nonpermissive cell cannot replicate in the target cell, but a Vif$^-$ virus produced in a permissive cell can (1). Borman et al. transfected HeLa cells to generate both wild type and Vif$^-$ viruses, which were used to infect different transformed T cell lines (1). The Vif$^-$ mutants from nonpermissive cells were 25-100 times less infectious than wild type Vif (1). After one round of Vif$^-$ mutant infection in nonpermissive cells, normal quantities of virus were released, but were noninfectious (1).

Evidence shows that Vif affects late events in the virus life cycle, such as virion assembly, budding, or maturation (12). Double-label immunofluorescence analyses of HIV-1 infected cell, performed by Simon et al. , indicate that the Vif and Gag proteins colocalize at the plasma membrane as virion assembly and budding occur, thereby allowing Vif to mediate these steps in the virus life cycle (12). Mutagenesis studies by Goncalves et al. show that basic domains in Vif's C terminus are necessary for membrane association (5). Disrupting this membrane association also inhibits HIV-1 replication. Thus, the membrane localization of Vif may be critical to its biological activity (5).

In another study, Yang et al. found that Vif is phosphorylated and regulated by p44/42 mitogen-activated protein kinase (MAPK) on Ser^{165} and Thr^{96} residues (14). Mutations in the Thr^{96} site, as well as other phosphorylation sites not regulated by MAPK, resulted in the loss of Vif activity and inhibition of HIV-1 replication (14). These findings indicate that the phosphorylation may induce a conformational change necessary for its biological activity, mediate its membrane association, or promote its interaction with the Gag protein, all affecting the assembly of the virus (14). The study also indicates that without Vif, virions can begin reverse transcription, but cannot complete it (14).

Similarly, the study by Borman et al. also notes that Vif$^-$ mutants are incapable of DNA synthesis in target cells, suggesting that reverse transcription is initiated, but is inefficient (1). A paper by Bryan Cullen further supports the notion that reverse transcription fails to go to completion, although the exact stage at which failure occurs is debated (3). After reaching the nucleus, little or no full-length provirus is

produced, and the partial transcripts are degraded (3). Thus, Vif is needed for the prolonged accumulation of reverse transcripts in HIV-1 infected cells, and its absence allows for their degradation (13).

Another report showed that Vif might act to inhibit HIV-1 protease, thereby preventing premature cleavage of certain structural proteins (8).

With all the speculation as to exactly what Vif's role is, structural information may be able to give researchers a clue to Vif's function. Since Vif exists as aggregates in solution, structural studies using NMR are not feasible. The protein must be monomeric in solution. Past studies have shown that making mutations in a protein can change its aggregation behavior (6). Hence, deletion mutants may be the key to determining the structure of Vif.

Materials and Methods

The bacterial expression plasmid pD10Vif, obtained from Dana Gabuzda of Harvard University, has the full-length wild type Vif gene fused with an N-terminal 6-His tag. PD10Vif was used to express the mutant proteins created. The PCR primers contained non-homologous 5' regions that encode the restriction enzyme sites Bam-H1 and Hind III (see Results). These sites match those on pD10Vif, allowing for easy insertion of the mutant gene into the vector, which was then transformed into bacterial cells to express protein.

PCR: 1µl of template (200ng/µl), .8µl of dNTP's, 1.5µl of primer 1, 1.5µl of primer 2, 10µl of 10X buffer and 82.5µl of sterile water were pipetted into an eppendorf tube, and put in the thermal cycler. After window 1 of PCR, the tube was microfuged, .5µl of taq polymerase was added, and the tube was microfuged again and put back into the thermal cycler until PCR was complete.

To ensure that the PCR product was there, a 2% agarose gel was run. The gel was made from 60ml of 10X TAE, .72g of agarose, and 3µl of Ethidium Bromide. Note: all 2% agarose electrophoresis gels were run for 30 minutes at 100 volts.

Restriction Digests: Restriction digests were performed on both the PCR product, and the pD10Vif vector. For the first digest, 40µl of PCR product, 5µl of 10X buffer E, 1µl of BSA, 2µl of Bam-H1, and 2µl of Hind III were added to the tube for a total volume of 50µl. For the second digest, 3µl of the

pD10Vif vector, 1µl of BSA, 5µl of 10X buffer E, 37µl of water, 2µl Bam-H1, and 2µl of Hind III were added to the tube for a total volume of 50µl. Both tubes were put in a hot water bath at 37°C for 3 hours.

Another 2% agarose gel was run to make sure that the vector was cut. The digested vector was compared to the uncut vector.

Dephosphorylation of 5' ends using CIAP: 10µl of 10X CIAP (calf intestinal alkaline phosphatase) reaction buffer, 2µl of CIAP, 63µl of water, and 25µl of the digested vector were added to a tube and incubated for 30 minutes at 37°C. Then 1µl more of CIAP was added, and the tube was incubated for an additional 30 minutes at 37°C. 2µl of .5M EDTA was added to stop the reaction, and the tube was heated at 65°C for 20 minutes.

The vector was purified using the Promega Wizard® Plus Minipreps DNA Purification System protocol. The purified vector was then run on another 2% agarose electrophoresis gel. The vector restriction digest with CIAP treatment had to be repurified with the Promega Wizard® Plus Minipreps DNA Purification System. The PCR product was also purified using the Promega PCR Miniprep protocol. A 2% agarose gel was run.

Another restriction digest was performed on the vector, as previously explained, a 2% agarose gel was run, and the DNA was cut from the gel and placed in a 1.5ml tube so that gel purification could be performed. 1ml of Wizard® Minipreps DNA Purification Resin was added to the tube with the gel and incubated at 65° for 5minutes, until the gel had melted. Purification using the vacuum manifold was performed as indicated by Promega protocol. Another 2% agarose gel was run to see if the vector was present.

Ligation: Ligation reactions were performed using 2 different purified vectors. The control contained 4µl of vector, 1µl of Buffer, 1µl of Ligase, and 4µl of water. The purified vector prepared by Sanna Hakansson was split into two different tubes. One contained 1µl vector and 7µl of PCR (SV1), while the other contained 3µl of vector and 5µl of PCR (SV2). Both reactions contained 1µl of Buffer and 1µl of Ligase. The other purified vector, prepared by myself, was prepared the same way as described above (CV1 and CV2, respectively).

Transformation: Plates were already prepared containing LB agar and Ampicillin. Frozen JM109 Competent Cells were removed from -70°C and placed on ice until thawed. 10µl of ligated cells were added to the JM109 cells and placed on ice for 30 minutes. The cells were heat-shocked in a hot water bath for 45-50 seconds at 42°C and placed on ice for 2 minutes. 900µl of room temperature SOC medium was added to each transformation reaction and incubated for 60 minutes at 37°C with shaking. For each tube, 100µl of cells were plated and incubated at 37°C for 12-14 hours.

20µl of Ampicillin were added to each of two flasks containing 20ml of LB agar. The flask were inoculated with colonies from plates SV1 and CV2 and allowed to shake over night at 37°C. These cells were then purified using the Promega Wizard® Plus Minipreps DNA Purification System for 5-10ml of bacterial culture.

A restriction digest was performed using 5µl of DNA, 2µl of Bam-H1, 2µl of Hind III, 2µl of BSA, 2µl of Buffer E, and 7µl of water. A 2% agarose gel was run first using dye, and then without using dye.

Two flasks were re-inoculated using colonies from plates SV2 and SV1, as previously described. The plasmid prep was performed using the Promega Wizard® Plus Minipreps DNA Purification System protocol. Restriction digests were performed on SV2 and SV3 as previously described, and a 2% agarose gel was run to see if the insert was present.

The plasmid prep SV3 was prepared for sequencing. The sample was dried in the speed vac, and 20ul of water were added to dilute the sample to 250ng/ml. The sample was then sequenced by Scott Grayburn in NIU's DNA Synthesis and Sequencing Lab.

The pD10Vif vector was re-cut using the restriction digest protocol discussed previously. A 2% agarose gel was run, and gel purification was performed using the Promega purification procedure previously mentioned. Another ligation and transformation were performed. A colony from plate #1 was inoculated into a flask containing 20µl Ampicillin and 20ml of LB. The cells were purified using the Promega Wizard® Plus Minipreps DNA Purification System protocol for 5-10ml of bacterial culture. A restriction digest was performed and a 2% agarose gel was run.

RESULTS

The primers used for PCR both contain Bam-H1 and Hind III restriction sites. The sequence of primer #1 is 5' GGC**GGATCC**ATTCATCTGTATTACTTT 3'. The sequence of primer #2 is 5' GGGG**AAGCTTC**TAGTGTCCATTCAT 3'. The bolded segments represent the Bam-H1 and Hind III restriction sites, respectively. The underlined segments are where the sequences begin.

Vif is 192 amino acids long, but the mutant constructed contains a deletion of the first 106 amino acids. Thus, the Vif mutant starts at amino acid 107 and ends with amino acid 192. The sequence of the final, desired protein would contain methionine (ATG) as the start site, followed by the 6-His tag, the amino acids Argenine (R), Glycine (G), and Serine (S), and finally, the mutant Vif sequence.

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                        #107                                    #120
ATG—6-His—R—G—S—I—H—L—Y—Y—F—D—C—F—S—D—S—A—I—R—K—A—L—L—
              #130                       #140                        #150
G—H—I—V—S—P—R-C—E—Y—Q—A—G—H—N—K—V—G—S—L—Q—Y—L—A—L—A—
                          #160                        #170
A—L—I—T—P—K—K—I—K—P—P—L—P—S—V—T—K—L—T—E—D—R—W—N—K—P—
    #180                              #190
Q—K—T—K—G—H—R—G—S—H—T—M—N—G—H
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Figure 1 shows the first 2% agarose gel run, and allows one to make sure that the desired product is present. Lane 1 contains the λ DNA/EcoR1+Hind III molecular weight marker, while lane 2 contains the PCR product. The whited in band in lane 2 has a molecular weight of <1.375Kb, indicating that the desired product is present. (Note: the same λ marker was used for each gel. Each band of the marker represents the molecular weights labeled in Fig.1)

Figure 2 shows the gel run after the first restriction digest. Lane 1 contains the λ marker; lane 2 contains the digested pD10Vif vector; and lane 3 contains the uncut plasmid, allowing for a comparison between the cut and uncut vector. Lane 2 indicates a clean cut. The cut plasmid ran slower (~10Kb) compared to the supercoiled, uncut plasmid in lane 3 (~7Kb). A second band is also present in lane 2 at ~1.375Kb.

Figure 3 shows the gel run after CIAP treatment and vector purification, in order to obtain the concentration of the purified vector. Lane 1 contains a dilution of the λ marker; lane 2 contains the vector; lane 3 contains the un-diluted λ marker; lane 4 contains the digested PCR product; and lane 5 contains a

ladder marker. Although not visible in this figure, the original photograph showed the ladder marker in lane 5, as well as the PCR digest in lane 4 (<1.375Kb). However, the vector in lane 2 was not present, calling for a re-purification of the vector. The PCR digest was purified as well. Another gel was run, and again the vector was lost.

Because the vector was not showing up on the gels, another restriction digest was performed. This digested vector was run on a gel, and an alternative purification method was used—gel purification—where the band is cut out of the gel. However, this method was also unsuccessful, as the vector was once again lost when run on a gel.

Although the vector was not visible, it may have still been present. Perhaps the concentration was not high enough to see on a gel. Hence, a ligation and transformation were performed. Two different vectors were used in two different ligations. One was purified by Sanna Hakansson (SV1 and SV2), and the other was purified by myself (CV1 and CV2). Different amounts of vector and PCR product were put into four different tubes—SV1, SV2, CV1, and CV2. The ligated vector and insert were transformed into JM109 cells. Plates SV1 and CV2 showed growth and were used to inoculate flasks containing LB and Ampicillin. Only flask SV1 showed growth after shaking over night at 37°C. The DNA was purified, and a gel was run. The Bam-H1/Hind III fragment was not seen at the right size, which is ~300 bases. Another gel was run without dye to see if a band could be distinguished at 300 bases. Again, no band was visible on the gel. Flasks were re-inoculated using colonies from plates SV1 and SV2. A purification was performed, restriction digests were done, and another gel was run (Figure 4). Lane 1 in figure 4 contains the λ marker; lane 2 contains 5μl of the digest SV1; lane 3 contains 5μl of the digest SV2. No bands were visible, so 15μl of the digests were loaded onto the same gel. Lane 4 contains the λ marker; lane 5 contains the digest SV1; lane 6 contains the digest SV2. As indicated by the whited in band in Fig.4, the insert was seen at 300 bases.

Figure 5 is an electroferrogram showing the sequence obtained from NIU's DNA Synthesis and Sequencing Lab. Unfortunately, it appears as though the PCR products and vector self-ligated. The pD10Vif vector was re-cut, a gel was run, and a gel purification was performed. Another ligation and

transformation were performed. Plate #1 showed growth and was inoculated into a flask containing LB and Ampicillin. The cells were purified, a restriction digest was performed, and a gel was run. Again, the vector was not visible.

CONCLUSION

Though the experiments did not work, I learned a lot about lab technique and research methods. I also learned to work with other people to troubleshoot to try to figure out where something may have gone wrong, and how sterile technique plays a large role in the success of an experiment.

I still believe that further research should be conducted concerning Vif. Making deletion mutants is vitally important to making Vif monomeric in solution, and ultimately allowing for a structure to be obtained using NMR spectroscopy. Determining the structure of Vif could give clues as to what Vif does, and how it does it. This could lead to new and better drug therapies.

Since HIV can integrate into the host's genome and undergo a period of latency, it is important to find ways to delay the symptoms and keep the virus from proliferating. The HIV life cycle contains more than a dozen steps. Thus, interrupting any one of these steps could prevent the virus' detrimental affects (2).

For example, AZT works to stop the reverse transcription of RNA into DNA, which would then integrate into the host cell's genome (2). Various protease inhibitors stop protease from cutting the proteins before a new virus can assemble. The problem with these drugs has been that the virus mutates into a strain that is resistant to the drug (2). The HAART drug combines both protease and reverse transcriptase inhibitors, which significantly decreases the viral load since the virus must overcome 2 barriers instead of just one in order to become resistant. If Vif's mechanism is found, a drug could be designed to prevent Vif from functioning properly, and consequently, prolong many lives.

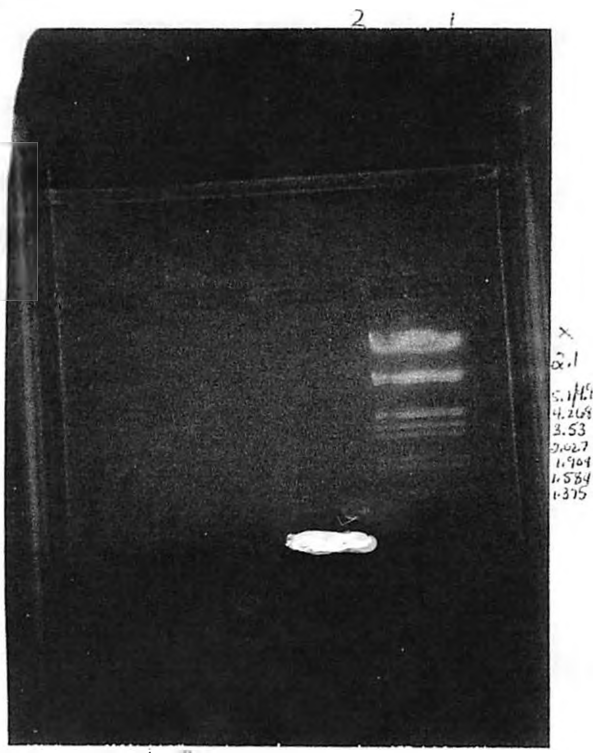


Figure 1

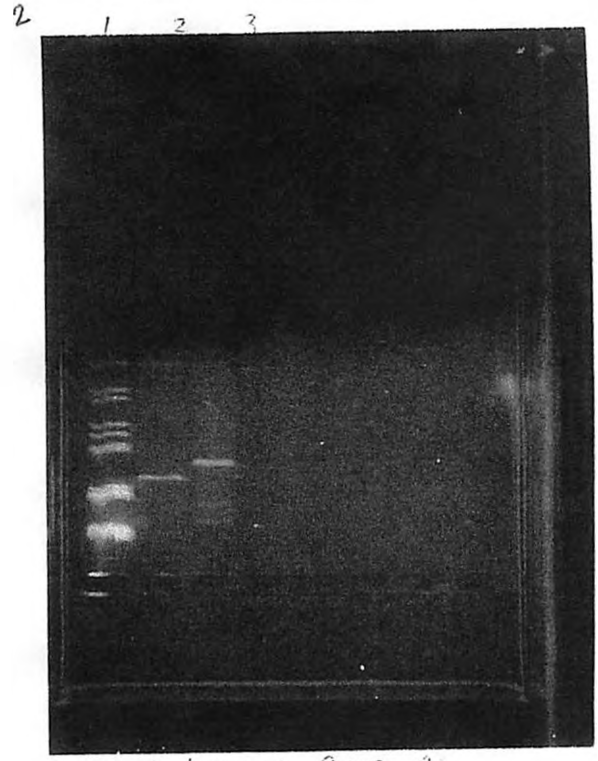
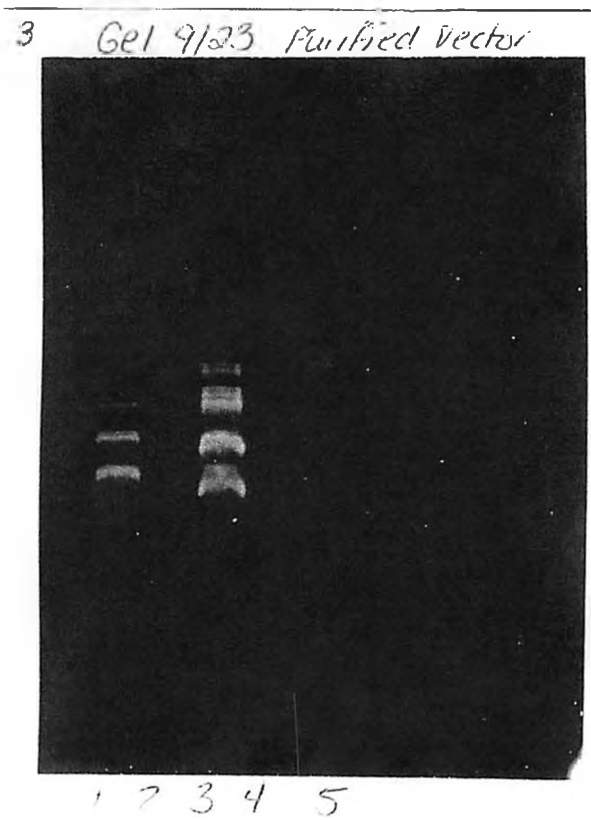


Figure 2

Figure 3

Figure 4



Model
Version 2.1.1

991307

5
Lane 5

Signal A:276 T:144 G:206 C:100
DYEnamic™ ET Term. {AnyPrimer}
DYEnamic™ ET Terminators
Points 640 to 6400 Base 1: 640

Fri, Dec 10, 1999 9:47 AM
Thu, Dec 9, 1999 3:39 PM
Spacing: 12.15

His tag

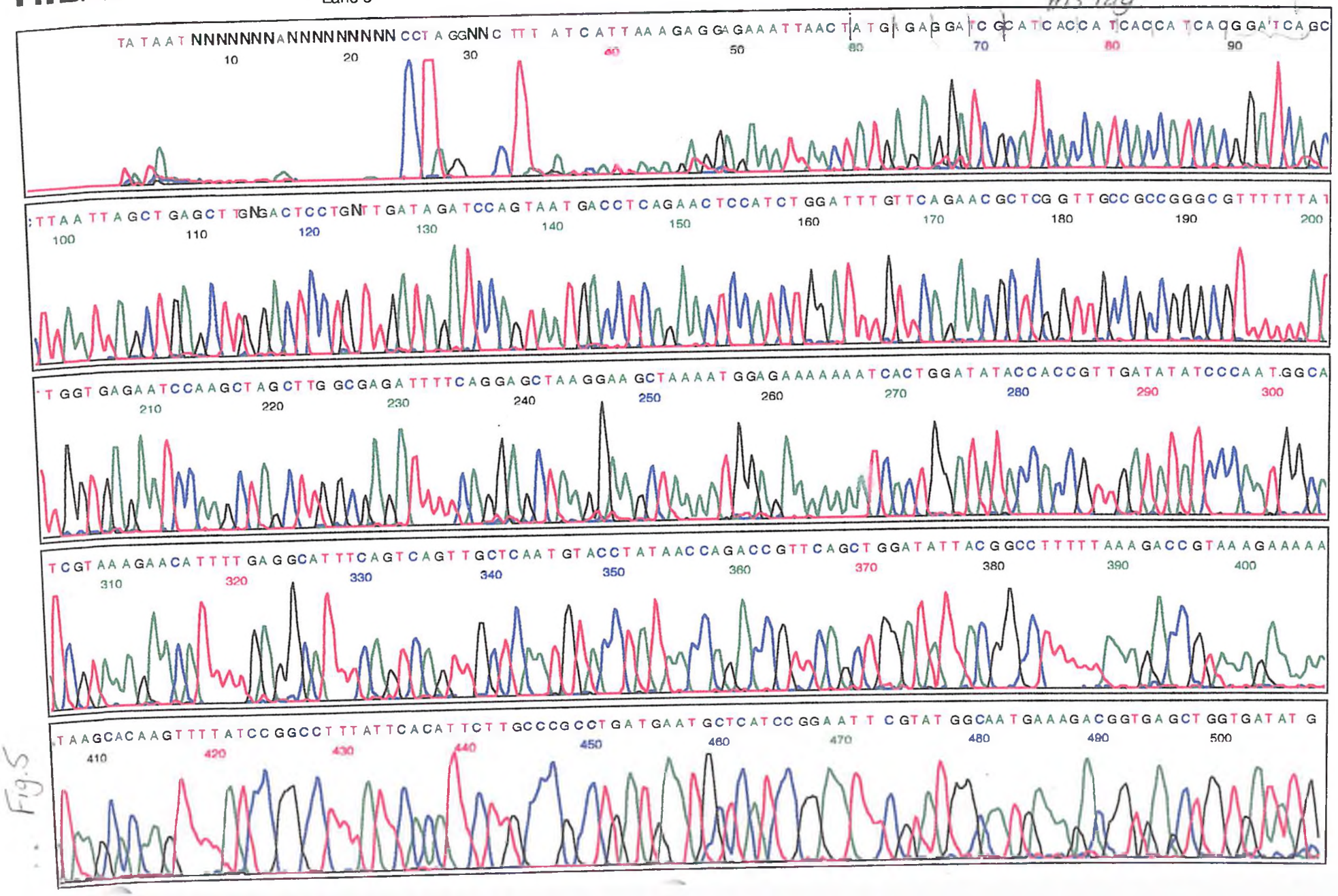


Fig. 5