5-1993

A Comparison of Hemoglobin Zurich Erythrocytic Membrane Proteins & Identification of the Mutation at the DNA Level

Yijuan Liu
Western Kentucky University

Liu,

Yijuan

1993

A COMPARISON OF HEMOGLOBIN ZURICH ERYTHROCYTIC MEMBRANE PROTEINS AND IDENTIFICATION OF THE MUTATION AT THE DNA LEVEL

A Thesis

Presented to

the Faculty of the Department of Biology

Western Kentucky University

Bowling Green, Kentucky

In Partial Fulfillment of

the Requirements for the Degree

Master of Science

by

Yijuan Liu

May,1993

AUTHORIZATION FOR USE OF THESIS

Permission is hereby

☒ granted to the Western Kentucky University Library to make, or allow to be made photocopies, microfilm or other copies of this thesis for appropriate research for scholarly purposes.

☐ reserved to the author for the making of any copies of this thesis except for brief sections for research or scholarly purposes.

Signed: Yijun Li

Date: 4-1-93

Please place an "X" in the appropriate box.

This form will be filed with the original of the thesis and will control future use of the thesis.

WKU—Printing paid from state funds, KRS 57.375.

A COMPARISON OF HEMOGLOBIN ZURICH ERYTHROCYTIC MEMBRANE PROTEINS AND IDENTIFICATION OF THE MUTATION AT THE DNA LEVEL

Recommended MARCH 9, 1993
(Date)

Martin R. Houston
Director of Thesis

Claire A. Rinehart

Frank Lomon

David R. Hartman

Frank Conley 3-9-93
College Dean (Date)

L. S. Alexander 3-29-93
Graduate Studies (Date)

ACKNOWLEDGMENTS

I would like to express my sincere gratitude and deep appreciation to my advisor, Dr. Martin R. Houston, for his guidance, material assistance and special patience. His dedication and professional input made my two years of graduate work enjoyable.

I am grateful to the other members of my committee, Dr. Claire A. Rinehart, Dr. David R. Hartman and Dr. Frank R. Toman for their help throughout the duration of my studies.

Appreciation is extended to Dr. Donella J. Wilson and Dr. Pilar Aguinaga for their advice and instruction. Thanks are also due to Dr. Nancy S. Dawson and Mr. Rodney R. McCurry for their photographic assistance.

I would also like to thank my husband, De Chen, and my parents, because without their love and support none of this research would have been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

A COMPARISON OF HEMOGLOBIN ZURICH ERYTHROCYTIC MEMBRANE PROTEINS AND IDENTIFICATION OF THE MUTATION AT THE DNA LEVEL

Yijuan Liu May 1993 31 pages

Directed by: Dr. M.R. Houston, Dr. C.A. Rinehart, Dr. D. R. Hartman and Dr. F.R. Toman

Department of Biology Western Kentucky University

Hemoglobin Zurich is a rare blood condition which causes severe rupturing of erythrocytes upon the administration of sulfonamides. Hemoglobin Zurich is caused by a specific genetic mutation in which there is a single amino acid (histidine) substituted for arginine at position 63 in the beta chain of hemoglobin molecules [ß 63 Histidine---> Arginine].

In this study, the membrane proteins of Hemoglobin Zurich erythrocytes were compared to membrane proteins from normal human erythrocytes employing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by periodic-acid Schiff (PAS) and DZANDU staining. No differences were detected employing these techniques.

DNA was isolated from leucocytes of a Hemoglobin Zurich individual, amplified and subsequently sequenced by the dideoxynucleotide method. The actual mutation was identified as a substitution of a G at base 188 of the coding region for an A.

INTRODUCTION

The development of acute hemolytic anemia during sulfonamide therapy was first described by Harvey and Janeway [1] in three patients receiving sulfanilamide for a bacterial infection. One year later Wood [2] emphasized the frequency of the phenomenon by reporting that acute hemolytic anemia developed in 21 of 522 patients treated with sulfanilamide. In 1960, Hemoglobin Zurich, the first of the abnormal hemoglobins to be associated with sulfonamide induced hemolysis, was discovered by Hitzig and his associates [3] in a Swiss family. Other cases were subsequently encountered in Maryland [4] and Japan [5]. The structural abnormality of Hemoglobin Zurich involves the position in beta chains corresponding to the distal histidine (ß63), which is substituted by arginine in the abnormal protein [6]. Individuals having Hemoglobin Zurich have been shown to be heterozygous but do not exhibit hemolytic anemia until exposed to sulfonamides [1]. The importance of searching for an abnormal hemoglobin in subjects with a sulfonamide-induced hemolytic anemia is evident.

Hemoglobin Zurich (histidine E7(63)ß--> arginine) is unique because it has a high oxygen affinity, low

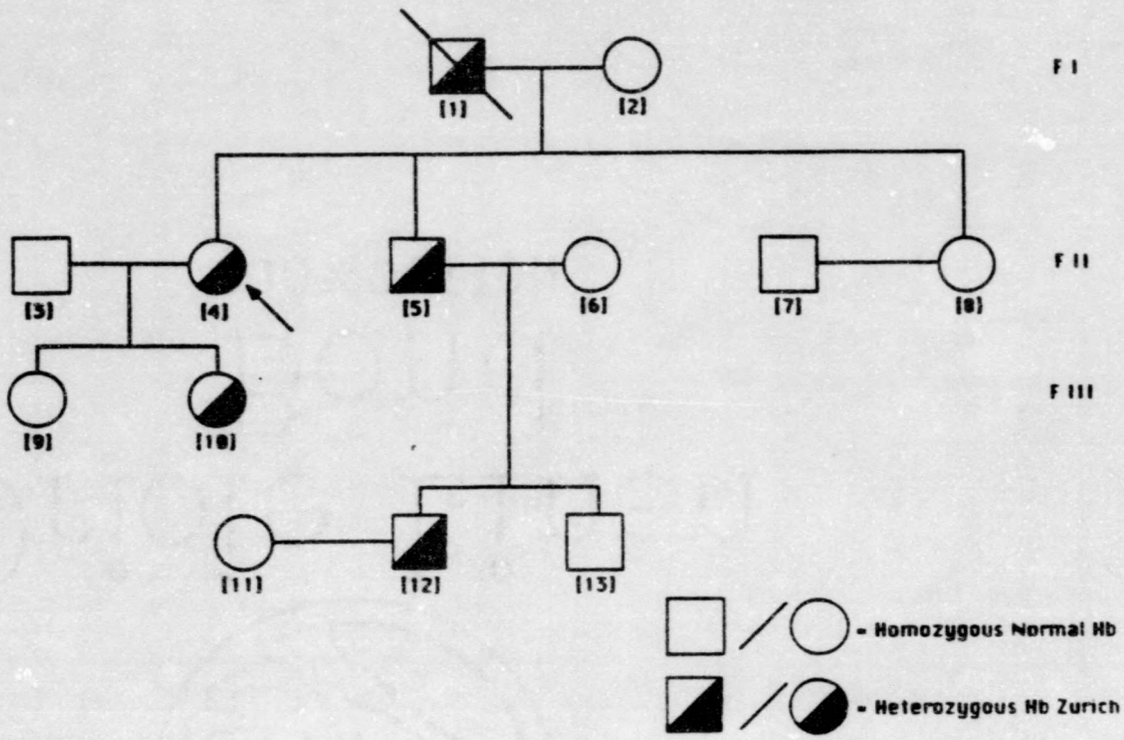
cooperativity and a normal Bohr effect; *in vitro*, the ß hemes are autoxidized more easily than those of Hemoglobin A [7]. Heinz bodies are induced only after sulfanilamide treatment. X-ray analysis has shown that the guanidinium group of arginine E7(63)ß forms a salt bridge with the propionate side chain of the heme [8]. Several other side chains edge in to fill the gap left by the histidine, but it remains large enough to accommodate a sulfanilamide molecule, allowing its sulfonamide group to form a hydrogen bond with the heme propionate and its amino group to act as an electron donor to the iron-bound oxygen.

The Hemoglobin Zurich condition of the family studied in this investigation was detected in 1965 and has not been previously reported in the literature. A female patient, approximately two months pregnant, developed a urinary tract infection during the late part of January, 1965, and was treated with sulfadyne. After four days, she became jaundiced and then developed aching in the neck, nausea, spots in the front of her eyes, and dark urine. Other family members were screened for the abnormal hemoglobin with the results shown in the pedigree chart, Figure 1. Individual 3, the normal homozygous individual, was designated as the control. Individuals 4, 5, 10 and 12 were selected as the experimental heterozygous Hemoglobin Zurich individuals.

The purpose of the current study was two-fold. First, the membrane proteins of Hemoglobin Zurich erythrocytes were

compared to those of normal human erythrocytes by SDS-PAGE, using PAS and DZANDU staining procedures. Secondly, the DNA of leucocytes was isolated, amplified and subsequently sequenced employing the dideoxynucleotide method [9].

Figure 1. Pedigree chart of the Hemoglobin Zurich family.


F I
F II
F III
[1]
[2]
[3]
[4]
[5]
[6]
[7]
[8]
[9]
[10]
[11]
[12]
[13]
- Homozygous Normal Hb
- Heterozygous Hb Zurich

MATERIALS AND METHODS

A COMPARISON OF ERYTHROCYTIC MEMBRANE PROTEINS FROM HEMOGLOBIN ZURICH AND NORMAL INDIVIDUALS

Isolation of Red Blood Cell Membranes

Blood specimens were obtained from four subjects with Hemoglobin Zurich who were members of one family. The control subject was a healthy adult family member. Venous blood was obtained in EDTA-vacuum tubes and stored on ice until studied, which was usually within two to four hours. Fresh blood was centrifuged at 3000 x g for 15 minutes, and the buffy coat was transferred and stored at -20°C until used to isolate and sequence DNA from Hemoglobin Zurich subjects.

The method of Steck *et al.*[10] was used to isolate the erythrocytic membranes. Intact cells were centrifuged using a bench top clinical IEC centrifuge at a setting of 3000 x g for 15 minutes at 4°C. The erythrocytes were washed two times with Hank's Balanced Salt Solution [0.14 g/L $CaCl_2$, 0.4 g/L KCl, 0.06 g/L KH_2PO_4, 0.1 g/L $MgCl_2 \cdot 6H_2O$, 0.1 g/L $MgSO_4.7H_2O$, 8.0 g/L NaCl and 0.09 g/L $Na_2HPO_4 \cdot 7H_2O$] and once with PES [10 mM phosphate buffer (pH 7.5), 0.15 M NaCl, 1.0 mM ice cold EDTA].

Erythrocytes were hemolyzed by washing one volume of

packed erythrocytes with 29 volumes of fresh, cold PED [5.0 mM phosphate buffer (pH 7.5), 1.0 mM EDTA, 0.25 mM dithiothreonine (DTT)]. The hemolysate was centrifuged at 4.0°C using a SS34 Sorvall rotor for 13 minutes at 20,198 x g in 30-ml Corning glass centrifuge tubes. The supernatant was discarded, and the pellet was washed with PED buffer two to three times. The pellet contained the ghosts (purified erythrocytic membranes).

The ghosts were prepared for storage by adding one volume of 5.0 mM phosphate buffer (pH 7.5) to one volume of each ghost sample plus two volumes of 2x gel sample buffer (100 mM Tris, 4% SDS, 0.2% bromophenol blue). The sample was boiled for five minutes and either stored at -20°C or electrophoresed on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE).

Electrophoresis

A 10% SDS polyacrylamide gel solution contained 5.0 mL of 30% acrylamide-0.8% Bis, 1.85 mL of 3.0 M Tris buffer (pH 8.8), 0.15 mL of 10% SDS, 1.5 mL of 50% glycerol, 6.6 mL of double distilled water, 19 μL of 10% ammonium persulfate (APS) and 5.0 μL of N,N,N',N'-tetramethyl ethylenediamine (TEMED). An 18% SDS polyacrylamide gel solution contained 9.0 mL of 30% acrylamide-0.8% Bis, 3.33 mL of 3.0 M Tris buffer (pH 8.8), 0.27 mL of 10% SDS, 2.7 mL of 50% glycerol, 34 μL of 10% APS and 9.0 μL of TEMED. These two gel solutions were added to two separate chambers of a gradient apparatus to form a 10% to

18% gradient SDS polyacrylamide gel (16 cm x 16 cm x 0.15 cm). An aqueous 0.1% SDS solution was used to overlay the surface of the resolving gel. After allowing one hour for complete polymerization, the 0.1% SDS solution was removed and replaced by a stacking gel. The stacking gel solution contained 1.25 mL of 30% acrylamide-0.8% Bis, 0.625 mL of 2.0 M Tris buffer (pH 6.8), 0.1 mL of 10% SDS, 1.0 mL of 50% glycerol, 7.0 mL of double distilled water, 25 μL of 10% APS, and 5 μL of TEMED. The gel was then left for about one-half hour before use.

The concentration of proteins was determined by the BCA (Bicinchoninic acid) method [11]. Thirty micrograms of total protein from each sample were used in each lane for discontinuous SDS-PAGE. The samples were electrophoresed for 17 hours at 100v.

Staining Polyacrylamide Gels for Proteins

The gels were stained for protein with PAS (periodic acid, acetic acid and Schiff reagent) and DZANDU staining. Both staining procedures employed were those described by Fairbanks *et al.*[12]. The gels were placed in glass containers with enough fixing, staining or destaining solutions to suspend the gels and then placed on a rocking platform for an appropriate period of time at room temperature.

PAS Staining Procedure

The gel was fixed by soaking in a solution containing 25% isopropanol and 10% acetic acid for one hour, 10%

isopropanol and 10% acetic acid for another hour and 10% acetic acid for 20 minutes.

The gel was stained by soaking in 0.5% periodic acid for two hours; 0.5% sodium arsenite / 5.0% acetic acid for 30 minutes; and three additional times in 0.1% sodium arsenite / 5% acetic acid for 20 minutes, followed by 20 minutes in 5.0% acetic acid before being rinsed in double-distilled water to remove the acetic acid. Schiff's reagent (200 mL) was added, and the gel agitated overnight.

Destaining was accomplished by soaking the gel in 0.1% metabisulfite in 0.01 M HCl for three to five hours. The metabisulfite was discarded, and fresh metabisulfite was added two additional times. Lastly, the gel was placed overnight in a final staining solution containing 0.1% coomassie blue, 25% methanol and 7.5% acetic acid.

The gel was stored in 1.0% glycerol, then removed and photographed. Afterwards, the gel was dried for four hours using a BIO-RAD Model 583 Gel Dryer.

DZANDU Staining Procedure

The gel was placed in a 40% methanol / 10% acetic acid solution for one hour, and then washed twice for 30 minutes with 10% ethanol / 5.0% acetic acid. The gel was suspended in the oxidizer (3.4 mM potassium dichromate and 3.2 mM HNO_3) for 20 minutes and then washed three times for 30 minutes with double-distilled water which caused the background to disappear. The final wash water was decanted, and the gel was

suspended in 20 mM $AgNO_3$ for 20 minutes. The gel was washed three times for two minutes with double-distilled water. The gel was then placed in the DZANDU developer (0.28 M Na_2CO_3 and 0.008% paraformaldehyde). When the desired color was obtained, the developer was decanted and the gel rinsed with double-distilled water. A 10% acetic acid solution was added to the gel for one hour. The gel was additionally stained by adding a mixture of 0.1% coomassie blue, 25% methanol and 7.5% acetic acid for one hour, and destained with a 25% methanol / 7.5% acetic acid solution until the destaining solution remained clear. The destaining solution was changed at 30-minute intervals. The gel was removed and photographed. Afterwards, the gel was dried for four hours using a BIO-RAD Model 583 Gel Dryer.

ISOLATION AND SEQUENCING OF DNA

DNA isolation

The DNA isolation procedure was that described by Bardhan *et al.* [13]. The buffy coat previously isolated from 5.0 mL fresh blood was resuspended in 5.0 mL of TE buffer [0.01 M Tris-HCl (pH 7.6), 0.001 M EDTA (pH 8.0)], followed by the addition of 250 µL of 10% SDS, 10 µL of RNAase A (10 mg/mL), and incubated at 37°C for 30 minutes. Twenty-five microliters of Pronase E (25 mg/mL) were added, and the mixture was incubated at 37°C overnight. One milliliter of a 5.0 M sodium perchlorate solution was added to the mixture and

then 5.0 mL of phenol/chloroform (25:24) were added. The mixture was centrifuged at 1300 x g for 15 minutes at room temperature. The top layer was removed and placed in a clear 15-mL centrifuge tube and extracted again with equal volume of phenol/chloroform, then centrifuged at 1300 x g for 15 minutes at room temperature. Finally, the top solution was extracted once with chloroform/isoamyl alcohol (24:1) and centrifuged at 1300 x g for 15 minutes at room temperature. The top solution was removed and placed into another clear centrifuge tube, and two volumes of cold 100% ethanol were added. The resulting solution was centrifuged at room temperature at 5000 x g for five minutes. The supernatant was discarded, and the pellet was washed with 8.0 mL of cold 70% ethanol and then centrifuged at 5000 x g for five minutes. The supernatant was discarded, and the pellet was air-dried for five minutes. The pellet was resuspended in 1.0 mL of TE buffer (pH 8.0), then mixed overnight using a rotary mixer at 4°C. The resulting DNA was diluted 1/10 in double-distilled water, and the absorbance at 260 and 280 nm was measured to determine the purity of the isolated DNA.

Oligonucleotides (primer) purification

The purification of oligonucleotides was done as described by Bardhan *et al.* [13]. A 19% polyacrylamide/urea gel (45 cm long x 27 cm wide x 0.4 mm thick) was cast by placing together two clear glass plates with a spacer between them, and the plates clamped with an alligator clamp on each

side of the plates.

The 19% polyacrylamide/urea gel solution contained 31.5 g of urea, 37.5 mL of acrylamide stock solution [76% (w/v) acrylamide, 4.0% (w/v) N,N'-methylenebisacrylamide], 9.5 ml of 10x TBE [10.8% (w/v) Tris base, 5.5% (w/v) boric acid, 0.02 M EDTA (pH 8.0)] and 9.5 mL of double-distilled water. The acrylamide solution was warmed to 37°C to dissolve the urea and then cooled to room temperature. To the acrylamide solution, 0.45 mL of a freshly prepared solution of 10% APS and 35 μL of TEMED were added and mixed well.

The solution was drawn into the barrel of a 50-mL syringe, and the nozzle of the syringe was introduced into the space between the two glass plates. The acrylamide solution was expelled from the syringe, and the space almost filled to the top. No air bubbles or leaks were allowed. Immediately, the appropriate comb was inserted. The acrylamide was allowed to polymerize for one hour at room temperature. When polymerization was complete, the comb was removed. The upper and lower reservoirs were filled with 1x TBE. The gel was pre-run by loading a 10 μL aliquot of tracking dye solution containing xylene cyanol FF and bromophenol blue to the small well (about 2.0 cm) on the gel and electrophoresed at 2280v/100mA/60W until the gel's temperature was 50-55°C, and the tracking dye had separated.

The primer sample was prepared by adding 0.5 mL of double-distilled water to each of primer sample and vortexed

for three minutes. The primer sample was transferred to separate clean labelled test tube. The tubes containing original primer were washed with 0.5 mL of double-distilled water and added to the test tube containing the primer sample.

The volume of the primer sample solution was reduced by adding an equal volume of 1-butanol, vortexed for three minutes and centrifugation at 14 x g for one minute. The top layer (butanol/water) of each sample was discarded. This procedure was repeated several times until the volume of the primer sample solution was below 75 μL. The remaining 1-butanol was removed by adding an equal volume of ethyl ether, vortexing and centrifuging at 14 x g for one minute. The ethyl-ether layer was removed, and the primer sample solution was held at 65°C for five minutes to remove residual ethyl ether. The volume of primer sample solution was measured, an equal volume of formamide was added, and the solution was heated at 70°C for five minutes. The primer sample was loaded and electrophoresed under the same condition as pre-run until the primer sample had migrated approximately two-thirds the length of the gel. The oligonucleotide was visualized by UV shadowing. The gel was transferred to a piece of plastic wrap and placed on silica gel thin layer chromatography, then observed in a darkened room using a hand-held, long-wavelength ultraviolet lamp to visualize the DNA.

The desired primer sample was isolated from the gel by removing horizonal primer band using a sharp, clean scalpel.

The gel slices were transferred to six microfuge tubes and one milliliter of elution buffer (0.1% SDS, 0.5 M ammonium acetate and 10 mM magnesium acetate) was added to each tube. The slices were crushed with a disposable pipette tip by using a circular motion and pressing against the sides of the tubes. The tubes were sealed and incubated at 37°C for 12 hours in a shaker incubator. The primer sample was centrifuged at 14 x g for two minutes, then pooled and filtered using a 0.45 μm syringe filter to remove the gel particles. One-hundred microliters of elution buffer were added to the tube of the gel fragments and centrifuged at 14 x g for two minutes and filtered. The elution procedure was repeated with 500 μL of elution buffer. Enough 5.0 M NaCl solution was added to the primer sample solution to adjust the concentration to 0.055 M, and absolute ethanol was added to adjust the final concentration of ethanol to 70%. The mixture/solution was placed at -20°C for two hours.

The primer sample solution was centrifuged at 63,907 x g for two hours using a Beckman ultracentrifuge with a SW 60 rotor. The supernatant was discarded, and the pellet was washed in 70% ethanol. The pellet was centrifuged at 14 x g for 15 minutes. The supernatant was discarded, and the pellet was air-dried for five minutes and resuspended in 200 μL of double-distilled water. The primer sample solution was diluted 1:40 in double-distilled water, and the absorbance of the sample was measured at 260 and 280 nm to determine the

purity of the primer sample.

Gel analysis for isolated genomic DNA

A 0.7% agarose gel solution containing 0.7% agarose, 0.5x TBE and 0.53 μg/mL ethidium bromide was prepared and placed in a BRL Horizontal System gel apparatus. Nine microliters of the DNA samples, and 1.0 μL of loading buffer (10x TBE, 50% glycerol, 0.25% xylene cyanol, and 0.25% bromophenol blue) were mixed. The sample mixtures were loaded on the gel and run at 75v until the bromophenol blue tracking dye migrated approximately 2.0 cm from the bottom of the gel. The gel was removed and photographed.

PCR for isolated genomic DNA

The polymerase chain reaction (PCR) procedure was that described by Tabor *et al.*[14]. Target DNA was diluted to a concentration of 10 ng/μL with a total volume of 500 μL. Two 1.5-ml microcentrifuge tubes were labeled A and B. Ten microliters of dNTP mix (2.5 mM of each), 69.4 μL of double-distilled water, 22.5 μL of 10x PCR buffer [100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin (Sigma Chemical Company)], 0.75 μL of 2.0 pmoles primer CD_3 (ATC ACT TAG ACC TCA CCC TGT GGA GCC A) sense and 0.75 μL of 100 pmoles primer CD_6 (TCA TTC GTC TGT TTC CCA TTC TAA AC) antisense were added to Tube A. Primers CD_3 and CD_6 (purchased from The DNA Factory, San Diego, CA 92121) are primers that will bind complemetary sites flanking the ß63 codon. The total volume was 111.35 μL of Tube A. Half of the solution in Tube A

(55.65 μL) was transferred to Tube B. A 112.5-μL sample of isolated genomic template DNA was added to Tube A, and 1.12 μL of Thermus aquaticus (Taq DNA polymerase) was added to Tube B. Four 1.0-mL microcentrifuge tubes were labeled 1, 2, -D (no DNA) and -E (no Taq). From Tube A, 67.3 μL were removed to Tube 1, 2 and Tube -E, then two drops of nujol mineral oil were added to four 1.0-mL tubes, and each heated at 95°C for six minutes. A 22.7-μL solution from Tube B was removed and transferred to Tube 1, 2 and Tube -D, and each heated at 52°C for three minutes. Amplification was performed for 30 cycles (92°C for two minutes, 65°C for one minute, 72°C for 2.5 minutes).

Gel analysis for PCR products

A 1.5% agarose gel solution containing 1.5% agarose, 0.5x TBE and 0.53 μg/mL ethidium bromide was prepared, placed in the agarose gel apparatus, and nine microliters of each PCR product and one microliter of loading buffer were mixed and loaded on the gel then electrophoresed at 75 v until the bromophenol blue tracking dye was approximately 2.0 cm from the bottom of the gel. The gel was removed and photographed.

DNA Sequencing

DNA sequencing was accomplished by Dr. Pilar Aguinaga using the procedure described by Tabor *et al.*[14]. A 6.0% polyacrylamide urea gel (45 cm long, 22 cm wide and 0.4 mm thick) was made with 7.5 mL of acrylamide stock solution [38% (w/v) acrylamide, 2.0% (w/v) N,N'-methylenebisacrylamide]; 5.0

mL of 10 x TBE; 24.024 g of urea; and 30 mL of double-distilled water. The mixture was heated to dissolve the urea and then cooled to room temperature. Four-hundred microliters of a freshly prepared 10% APS solution and 20 μL of TEMED were added to the acrylamide solution, mixed, and the gel was allowed to polymerize for one hour at room temperature between the electrophoretic glass plates.

The DNA sample isolated from Individual 4 was subsequently prepared. Four 1.5-mL microcentrifuge tubes (termination tubes) for each sample were prepared and labeled A, C, G and T. Two and one-half microliters of dideoxy NTPs (ddATP, ddCTP, ddGTP and ddTTP) were added to its respective tube and held at 37°C. A DNA-containing tube was prepared containing 7.0 μL of PCR product, 2.0 μL of 5 x sequencing buffer [200 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 250 mM NaCl) and 2.0 μL of Zurich Sequencing Primer (5.0 pmol/μL). The sequence of the Zurich Sequencing Primer is TAC CCT TGG ACC CAG AGG TTC TTT (purchased from Meharry Medical College Corn Facility). The mixture (Mix 1) was heated to 65-70°C for two minutes then placed in ice. A mixture (Mix 2) was made containing 1.0 μL of labeling solution (15 μM dITP, 7.5 μM dCTP, 7.5 μM dTTP), 7.0 μL of double-distilled water, 4.0 μL of DTT and 4.0 μL of deoxyadenine-5'-α-[^{35}S] thiotriphosphate (1000/1500 ci/mmol). An enzyme mixture (Mix 3) was made with 1.0 μL of the Sequenase Version 2.0 enzyme (8000-30,000 units/mL) and 7.0 μL of enzyme dilution buffer (10 mM Tris-HCl

(pH 7.5), 5.0 mM DTT, 0.5 mg/mL BSA).

To a microfuge tube, 8.0 μL of Mix 1, 4.0 μL of Mix 2 and 2.0 μL of Mix 3 were added. The tube was microfuged 20 seconds to initiate the labeling reaction and held at room temperature for three minutes. Next, 3.5 μL of the centrifuged mixture was added to each termination tube (A, C, G, and T) and incubated for five minutes at 37°C. Four microliters of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) were added, and the tubes incubated for three minutes at 90-95°C for denaturization. The samples were stored on ice.

The 6.0% polyacrylamide urea gel was pre-run at 1500V/ 100mA/60W until the gel temperature was approximate 47°C. The wells were washed with TBE buffer to remove the urea. Then, 3.5 μL of the four experimental mixtures (A, C, G and T) were loaded into each of four wells. The gel was run at 1500V/100mA/60W until the bromophenol blue was about 2.0 cm from the bottom of the gel. Subsequently, 3.5 μL of the four experimental mixtures were added to four adjacent wells and electrophoresed under identical conditions until bromophenol blue was once again about 2.0 cm from the bottom of the gel.

The plates were carefully separated. The gel was placed on a mirrored glass plate and soaked in a 10% methanol/10% acetic acid solution for 15 minutes. Afterwards, the 10% methanol/10% acetic acid solution was siphoned off, and the plate was removed from the tray. Whatman paper was applied to

the gel, the gel plate inverted, and the paper with the gel attached was slowly removed as a unit.

The gel was covered with plastic wrap as evenly as possible and placed in a gel dryer under vacuum at 80°C for 45 minutes. The gel was removed from the gel dryer.

X-ray film (Kodak X-Omat AR) was placed on the surface of the gel in a X-ray film holder. The holder was closed and completely covered with aluminum foil and exposed 22 hours at -70°C. The film was developed using the developer solution for three minutes, water wash for two minutes and fixer for five minutes. The film was then washed with tap water and allowed to dry.

RESULTS AND DISCUSSION

A COMPARISON OF ERYTHROCYTIC MEMBRANE PROTEINS FROM HEMOGLOBIN ZURICH AND NORMAL INDIVIDUALS

The erythrocytic membrane proteins from four Hemoglobin Zurich individuals of the same family have been examined by electrophoresis and compared to a normal family member donor. The concentrations of the ghost membrane proteins isolated from the four Hemoglobin Zurich individuals are shown in Table 1. The range of concentrations of the isolated ghost membrane proteins from five subjects was from 0.28 to 0.66 $\mu g/\mu l$. The purpose of the electrophoresis was to separate the major membrane proteins and to visualize by the staining procedures. Examples of SDS-PAGE gels stained for ghost membrane proteins using PAS and DZANDU staining procedures are shown in Figures 2 and 3. Electrophoretic comparisons of the isolated proteins from Hemoglobin Zurich ghost membranes (Figures 2 and 3) indicated no obvious variations in the electrophoretic banding patterns employing the electrophoretic techniques used in this study. The high molecular weight polypeptides, Bands I and II, have reported values ranging from about 130,000 to 300,000 daltons [15]. The small components, V (41,300 daltons) and component VI (36,200 daltons) detected by Mitchell and Hanahan

TABLE 1

The concentrations of the ghost membrane proteins isolated from four Hemoglobin Zurich individuals

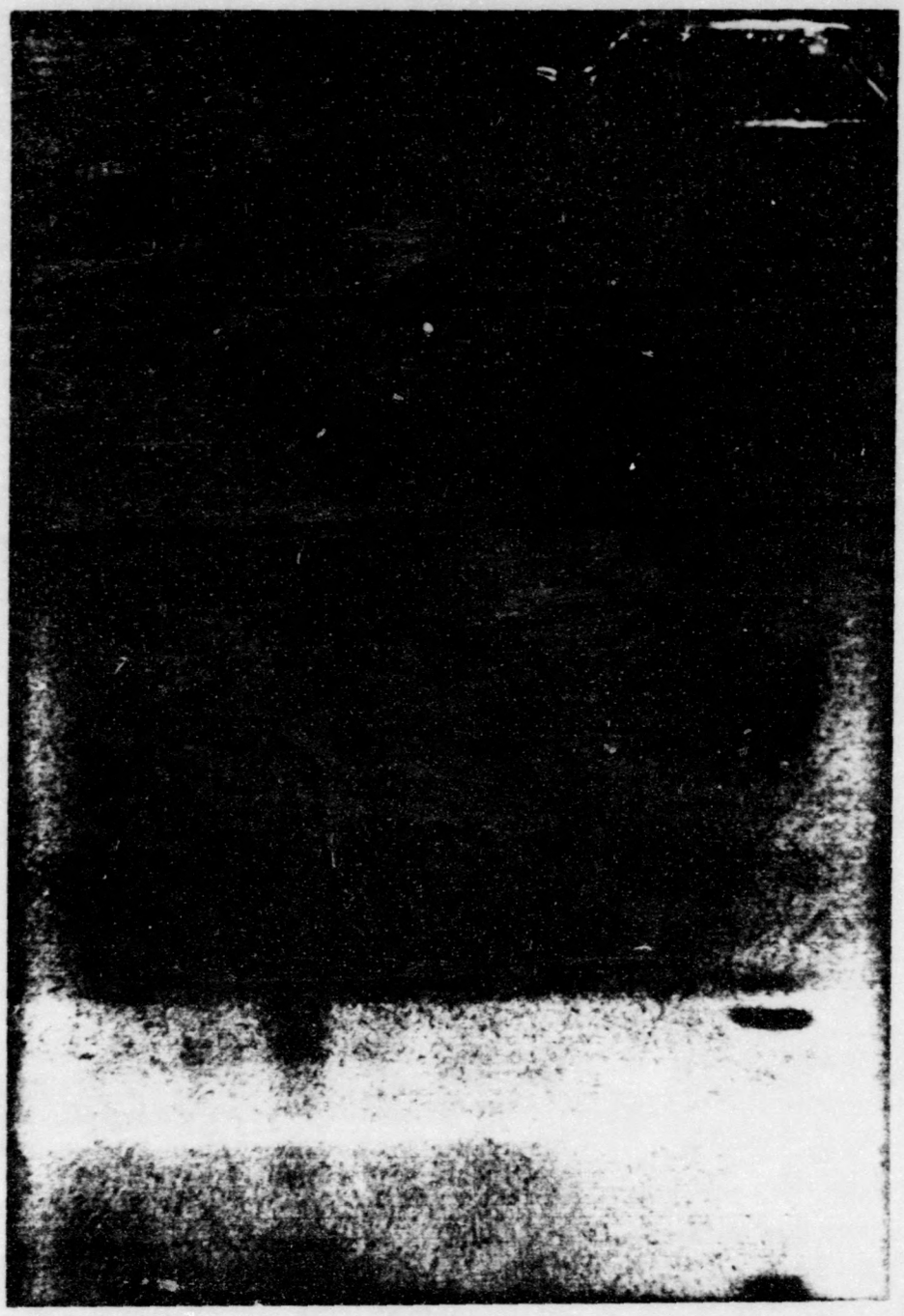
Samples	Membrane Proteins ($\mu g/\mu L$)
3*	0.66
4	0.31
5	0.28
10	0.29
12	0.52

* Control

Figure 2. SDS-PAGE gel stained for ghost membrane proteins using PAS staining procedure: L = low marker; H = high marker; lane 1 = Individual 3; lane 2 = Individual 5; lane 3 = Individual 4; lane 4 = Individual 12; and lane 5 = Individual 10. The molecular weights of the high marker from top to bottom are 200,000, 116,250, 92,500, 66,200 and 45,000 daltons; The molecular weights of the low marker from top to bottom are 92,500, 66,200, 45,000, 31,000, 21,500 and 14,400 daltons.


L
H
1
2
3
4
5

Figure 3. SDS-PAGE gel stained for ghost membrane proteins using DZANDU staining procedure: L = low marke; H = high marker; lane 1 = Individual 10; lane 2 = Individual 12; lane 3 = Individual 4; lane 4 = Individual 5; and lane 5 = Individual 3. The molecular weights of the high marker from top to bottom are 200,000, 116,250, 92,500, 66,200 and 45,000 daltons; The molecular weights of the low marker from top to bottom are 92,500, 66,200, 45,000, 31,000, 21,500 and 14,400 daltons.


1
2
3
4
5
H
L

[16] were apparent in the Hemoglobin Zurich membranes. Components III (89,000 daltons) and IV (77,500 daltons) appear to correspond to major bands recognized by Lenard [17, 18]. There appear to be no major differences in erythrocytic membrane polypeptides between the control and Hemoglobin Zurich individuals.

ISOLATION AND SEQUENCING OF DNA

The absorbances of isolated genomic DNA at 260 and 280 nm and concentrations of the DNA are shown in Table 2. The concentration of DNA was calculated using the equation:

$C = A_{260} \times 50\ (\mu g/\mu L) \times (1/df\ [\text{dilution factor}])$.

From the ratio of A_{260}/A_{280}nm, the isolated genomic DNA appeared to be almost pure [19].

The absorbances at 260 nm and 280 nm and the ratio of absorbance of 260 nm to 280 nm for the purified primer, AN_{11} (Zurich sequencing primer), and the concentration of primer are shown in Table 3. The concentration of primer was calculated using the equation: $C = A_{260}/E$ where E_{ATP} is 15.4 ml/μmol, E_{TTP} is 8.8 ml/μmol, E_{GTP} is 11.7 ml/μmol and E_{CTP} is 7.3 ml/μmol [20].

Data confirming the presence of isolated genomic DNA from the leucocytes (buffy coat) are shown in Figure 4. All samples from individuals contained DNA although the sample from individual 5 possessed the least. The amplification results are illustrated in Figure 5. After amplification,

TABLE 2

The absorbances at 260 and 280 nm and concentrations of isolated genomic DNA from normal control and Hemoglobin Zurich individuals

Samples	A_{260}	A_{280}	A_{260}/A_{280}	DNA ($\mu g/\mu L$)
3*	0.127	0.066	1.924	63.5
4	0.055	0.031	1.764	27.5
5	0.176	0.093	1.881	88.0
10	0.024	0.015	1.642	12.0
12	0.128	0.075	1.700	64.0

* control

TABLE 3

The absorbances at 260 nm and 280 nm, ratio of absorbance of 260 nm to 280 nm, and concentration of the primer

	Primer* AN_{11}
A_{260}	0.008
A_{280}	0.006
A_{260}/A_{280}	1.367
Concentration (μmol/ml)	3.30×10^{-5}

* AN_{11}: Zurich sequencing primer

Figure 4. Agarose gel analysis of isolated genomic DNA from Hemoglobin Zurich individuals and normal control: M = 1 Kb marker (GIBCO BRL); lanes 1 and 5 = Individual 3; lanes 2 and 6 = Individual 12; lanes 3 and 7 = Individual 5; lanes 4 and 9 = Individual 10; lane 8 = Individual 4.

M 1 2 3 4 5 6 M 7 8 9

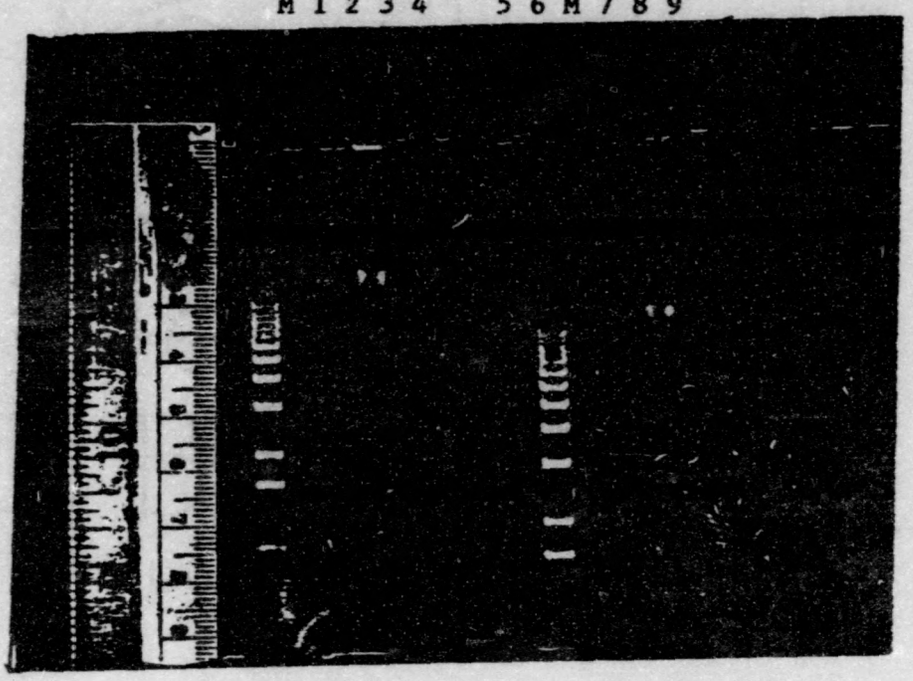
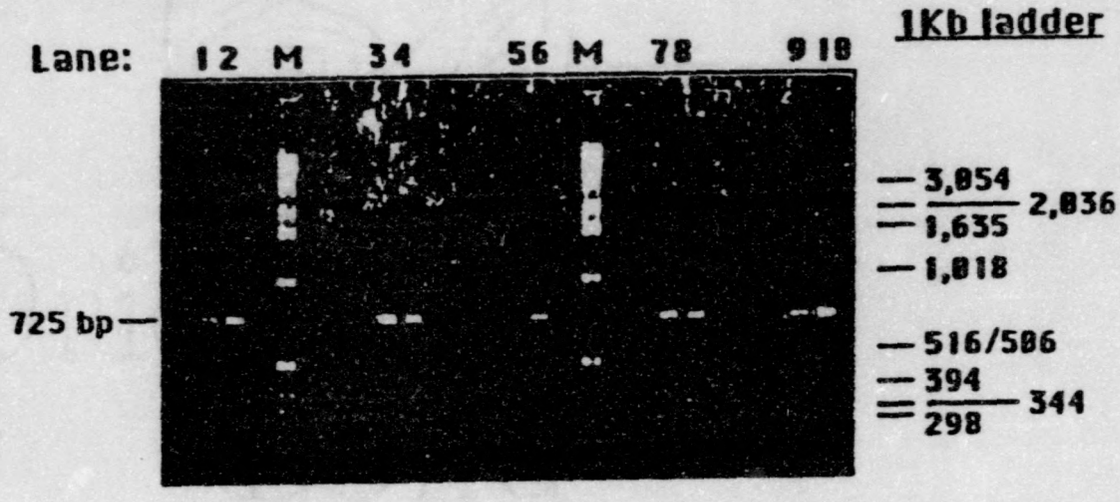


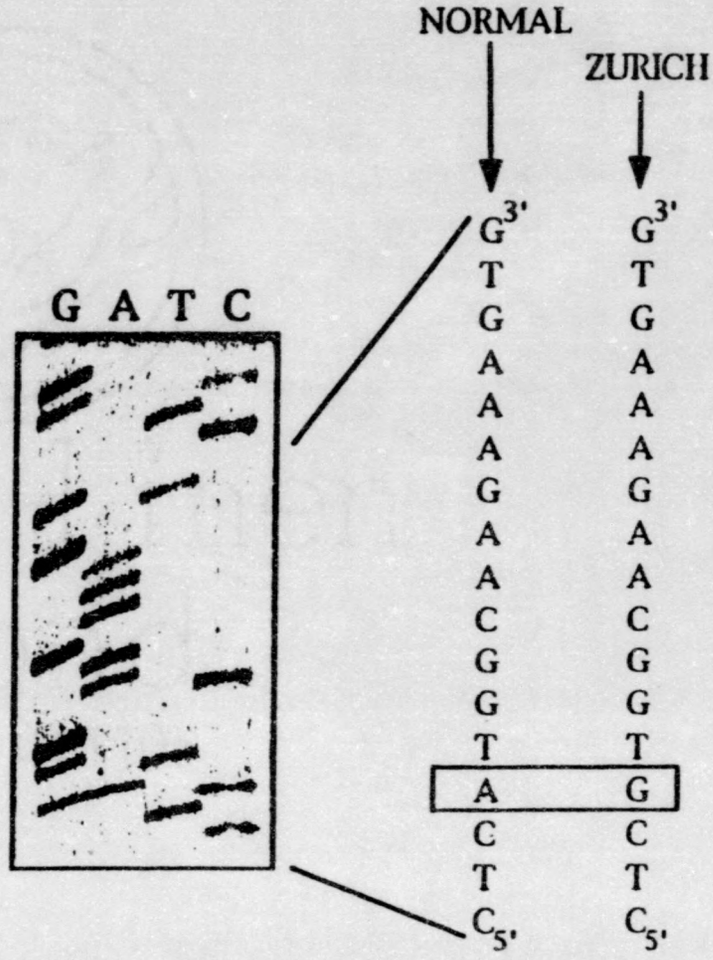
Figure 5. Agarose gel analysis of PCR products from Hemoglobin Zurich individuals and normal control (1/10 PCR products run on gel): M = 1 Kb marker; lanes 1 and 2 = Individual 3; lanes 3 and 4 = Individual 12; lanes 5 and 6 = Individual 5; lanes 7 and 8 = Individual 4; lanes 9 and 10 = Individual 10.


Lane: 1 2 M 3 4 5 6 M 7 8 9 10
1Kb ladder
3,054
2,836
1,635
1,018
725 bp
516/506
394
344
298

suitable quantities of DNA had been obtained for subsequent sequencing. An example of a DNA sequencing polyacrylamide / urea gel for Hemoglobin Zurich Individual 4 is illustrated in Figure 6.

Hemoglobin Zurich is clearly a most unusual hemoglobin variant because its abnormal ß chain has a different amino acid at position 63 [6]. The evidence is based on data obtained by sequencing the amplified DNA with a deoxyadenine-5'-α-[^{35}S] thiotriphosphate. As shown in Figure 6, the sequencing of the Hemoglobin Zurich Individual 4's PCR product revealed two bases at the position 188. The first 129 bases of the nucleotide sequence of the human ß-globin gene were provided by Dr. Pilar Aguinaga and the remaining decribed by Lawn *et al.* [21]. Base A was from the normal hemoglobin ß subunit DNA, and the other base G was the result of a point mutation in the Hemoglobin Zurich ß subunit DNA. No other mutations were identified from beginning of Zurich Sequencing Primer to sequence of 150 bases codon. The G to A substitution changes the codon of the ß subunit from histidine (CAT) to arginine (CGT). The DNA sequence of the remaining Hemoglobin Zurich individuals used in this study will be accomplished in the near future.

Figure 6. DNA sequencing gel of Hemoglobin Zurich individual 4.


NORMAL
ZURICH
G A T C
G 3'
T
G
A
A
A
G
A
A
C
G
G
T
A
C
T
C 5'
G 3'
T
G
A
A
A
G
A
A
C
G
G
T
G
C
T
C 5'

LITERATURE CITED

1. Harvey, A.M. and C.A. Janeway. 1937. The development of acute hemolytic anemia during the administration of sulfanilamide (para-aminobenzene- sulfonamide). J. A. M. A. 109:12.

2. Wood,W.B.Jr.. 1938. Anemia during sulfanilamide therapy. J. A. M. A. 111:1916.

3. Hitzig, W.H., P.G. Frick, K. Betke and T.H.J. Huisman. 1960. Hemoglobin Zurich eine neue hemoglobinanomalie mit sulfonamidinduzuierter innernkorperanemia. Helvt. Paediatr. Acta. 15:499.

4. Dickerman, J.D., N.A. Holtzman and W.H. Zinkham. 1973. Hemoglobin Zurich: A third family presenting with hemolytic reactions to sulfonamides. Am. J. Med. 55:638-642.

5. Murate, K., S. Yamamoto, Y. Hirano Mitsuhiro Omine, J. Tsuchiya, Y. Ohba and T. Miyaji. 1982. First Japanese family with the unstable Hemoglobin Zurich [ß63(E7)-->Arg]. Jap. J. Med. 21:140-145.

6. Bucci, E. and C. Fronticelli. 1965. A new method for preparation of α and ß subunits of human hemoglobin. J. Biol. Chem. 240:551.

7. Winterhalter, K.H., N.M. Anderson, G. Amiconi, E. Antonini, and M. Brunori. 1969. Functional properties of Hemoglobin Zurich. Eur. J. Biochem. 11:435-440.

8. Stamatoyannopoulos, G., A.W. Nienhuis, P. Leder and P.W. Majerus. The Molecular Basis of Blood Diseases. Philadelphia: W.B. Saunders Company, 1987, pp. 165-166.

9. Sanger, F., S. Nicklen and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

10. Steck, T.L., R.S. Weinstein, J.H. Strauss, and D.F.H. Wallach. 1970. Inside-out red cell membrane vesicles - preparation and purification. Science. 168:255.

11. Wiechelman, K., R. Braun and J. Fitzpatrick. 1988. Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. Anal. Biochem. 175:231-237.

12. Fairbanks, G., T.L. Steck and D.F.H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochem. 13:2606-2617.

13. Bardhan, S., C. Sharan, D.N. Singh, E.A. Turner, and M.D.P. Aguinaga. 1992. A simplified procedure for isolation of highly purified genomic DNA. Bionature, 12:IN PRESS.

14. Tabor, S. and C.C. Richardson. 1987. DNA sequence analysis with a modified bateriophage-T7 DNA polymerase. Proc. Nat. Acad. Sci. USA 84:4767-477.

15. Maechesi, S.L., E. Steers, V.T. Marchesi and T.W. Tillack. 1969. Red cell membrane, structure and function. Biochemistry. 9:50.

16. Mitchell, C.D. and D.J. Hanahan. 1966. Solubilization of certain proteins from human erythrocyte stroma. Biochemistry. 5:51.

17. Lenard, J.. 1970. Protein and glycolipid components of human erythrocyte membranes. Biochemistry. 9:1129.

18. Lenard, J.. 1970. Protein components of erythrocyte membranes from different animal species. Biochemistry. 9:5037.

19. Sambrook, J., E.F. Fritsch and T. Maniatis. Molecular Cloning. New York: Cold Spring Harbor Laboratory Press, 1989, pp. E.5.

20. Sambrook, J., E.F. Fritsch and T. Maniatis. Molecular Cloning. New York: Cold Spring Harbor Laboratory Press, 1989, pp. 11.30.

21. Lawn, R.M., A. Efstratiadis, C. Connell and T. Maniatis. 1980. The Nucleotide Sequence of the Human ß-Globin Gene. Cell. 21:647-651.