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Restriction fragment length polymorphisms in Southern African populations and a search for linkage to tyrosinase-positive oculocutaneous albinism

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2. SUBJECTS, MATERIALS AND METHODS

2.1 Subjects studied

2.1.1 Population screening and family studies

The author was fortunate to obtain the co-operation of a large number of individuals for the present study. Healthy volunteers from southern African San, Caucasoid and Bantu-speaking Negroid populations donated peripheral blood samples for population screening and for determination of allele frequencies. Pedigree analysis was carried out with the families of some of these individuals and with other consenting families.

Field trips were undertaken in order to seek out the more isolated populations such as the San of the Kalahari Desert. Figure 2.1 shows scenes from a typical field trip involving blood collection in the Tsumkwe district of the Kalahari.



Figure 2.1 !Kung San are depicted in these scenes from a field trip to Tsumkwe, Namibia.

The San population

The San of the Kalahari Desert are the most numerous of the surviving hunter-gatherers of Africa. Since an estimation of their numbers at 53 000 in 1955, their numbers have probably increased (Nurse and Jenkins, 1977). This indicates that there is no apparent danger of the biological extinction of the San, although their disappearance as the result of assimilation and intermarriage with other groups may be likely.

The San are a people generally short of stature, with a light, yellowish-brown skin colour, 'peppercorn' hair, small, often lobeless ears, and small faces with medial epicanthic folds around the eyes. Other distinctive features include the texture of the skin, which becomes deeply wrinkled and loose early in life, and their marked steatopygea.

The distinctiveness of the San and their striking similarity to the culturally different Khoi is demonstrated by their monogenic characters. There are a few serogenetic markers which appear to define the Khoisan peoples, such as the mis-named ABO allele, A^{bantu}, which is very common in the Khoisan and absent or very rare in the African Negro; the red cell acid phosphatase P^{r} allele (Jenkins and Corfield, 1972); and the $Gm^{1,13,15}$ and $Gm^{1,21}$ haplotypes (Jenkins *et al.*, 1970).

Other traits which are present in both San and Negro have been among the most powerful arguments for their common origin from a single stock (Singer and Weiner, 1963).

The genetic profile of the San has been well reviewed by Nurse *et al.* (1985). In the present study, the !Kung San were chosen both for their accessibility and as representative of the Khoisan peoples.

The Negroid population

The African Negroes have brown to brownish-black skin colour. They are of medium height, with broad noses and lightly curled black or brownish-black hair. In South Africa in recent years they have represented 72.0 per cent of the estimated 29 million individuals in the total population (Race Relations Survey, 1985).

There are several monogenic characters which appear to define the Negro populations, as well as those which are present in both the Negro and the Khoisan. The most striking Negro monogenic characteristics seem invariably to be introductions into the San rather than obvious indications of common ancestry. Among these are $PepA^2$ and $PepD^2$ at the peptidase A and D loci. These are found rarely in the San, who have a characteristic allele of their own, $PepD^3$ (Nurse *et al.*,

1985). A trait found almost exclusively in Negroes is the Gm(6) antigen, usually in the $Gm^{1,5,6}$ and $Gm^{1,5,6,14}$ haplotypes (Jenkins *et al.*, 1970; Jenkins, 1972).

There has been almost negligible gene flow from Caucasoids into the southern African Negro in modern times, and most of the genetic contribution to them by the San and Khoi cannot be dated accurately. This contribution is undoubtedly large, however; using the Gm genetic marker system, Jenkins *et al.* (1970) have determined a 10 - 50 per cent concentration of Khoisan genes in various Negro chiefdoms.

Nurse *et al.* (1985) have said that it is "well-nigh impossible to place the Southern African Negro in satisfactorily discrete and incontrovertible categories." (p. 164). There is inconsistent overlap of culture, historical tradition, and linguistic elements between groups. Rapid urbanization and social change have caused disruption of the family unit and breakdown of traditions and cultural mores, resulting in frequent marriages across ethnic barriers, especially in urban areas.

In the present study, a mixed group of accessible urban Negroes represents the Bantu-speaking southern African Negro populations.

The Caucasoid population

According to the Race Relations Survey of 1985, the Caucasoid population in South Africa comprised 15.6 per cent of the total population. South African Caucasoids are of mixed European stock: settlers in the 17th century were mainly from Low Germany or were French Huguenots; in later centuries immigrants continued to be Dutch and German. There was some non-Caucasoid gene flow into the community, including Malay, Khoi and Sinhalese. In the 1820s there was a significant influx of British settlers, and after the opening of diamond and gold mines in the late 19th century there were further settlers from Europe, some of these Jews from Poland and Lithuania.

The genetic profile of the South African Caucasoids has been found to be characteristic of the north-western European profiles (Nurse *et al.*, 1985). In the present study, the urban southern African Caucasoids investigated represent a sample with mixed European origins.

2.1.2 Linkage studies

Albinism

South African Negroid populations provided the subjects for the albinism study. Subjects were drawn from the large urban community of Soweto and from suburbs of the Johannesburg and Pr[illegible]ia metropolitan areas.

It was not possible to distinguish heterozygotes (Roberts *et al.*, 1986), so that a limited number of family members were useful for linkage. An additional problem was that in the prevailing conditions of social unrest, family members were often widely dispersed and could not be located. This resulted in the study families being of reduced size and often lacking a parent.

Forty-five Bantu-speaking Negroid families in which one or more family members had the tyrosinase-positive albinism phenotype were identified by E. Zwane, a member of staff of the Unit, on the basis of the clinical features described by Witkop *et al.* (1983). In fourteen families tested, the phenotype was confirmed by means of the hairbulb incubation test (Kugelman and van Scott, 1961; Witkop *et al.*, 1970; Kromberg, 1985). The remaining families were not tested for tyrosinase activity, since it became difficult to justify

obtaining the hairbulbs required for the test. Recent reports had concluded that the hairbulb incubation test is not consistent in distinguishing tyrosinase-negative from tyrosinase-positive individuals (reviewed in van Dorp 1987). In addition, no tyrosinase-negative individuals have been ascertained by any criteria in Southern Africa (Kromberg, 1985).

Non-paternity was excluded using the systems described in Section 2.8.1. The remaining families in the study were of two or three generations and included at least one affected and one unaffected child. Sibship sizes ranged from two to ten.

C.E.P.H.

Other linkage analyses were carried out using DNA samples from the Centre d'Etude du Polymorphisme Humain (C.E.P.H.), in a collaborative study with this Paris-based group (see Section 1.4.3). The DNA samples were extracted from the lymphoblastoid cell lines of forty families drawn from Caucasoid populations. Of these forty families, one was an Amish kindred, two were Venezuelan kindreds, ten were French and twenty-seven were Mormon kindreds from Utah. Thirty-one of the forty families were of three generations, twenty-eight families included four grandparents, and sibship sizes ranged from five to fifteen.

2.2 Materials

Deionised, distilled water was used throughout and all purchased chemicals were reagent grade.

2.3 Identification of Lambda Charon 4A recombinant bacteriophage containing single-copy human DNA

Human DNA libraries prepared from *Eco*RI and from *Hae*III/*Alu*I partial digestion of total human genomic DNA were obtained in bacteriophage lysate form as a gift from T. Maniatis (Maniatis *et al.*, 1978). Bacteriophage from these libraries were grown either upon the *Escherichia coli* host strain Q358 (*hsd* R_k^-, *hsd* M_k^+, *sup F*, *ϕ80*^{s}) or upon LE392 (F^-, *hsd R514* (r_k^-, m_k^+), *supE44*, *supF58*, *lacY1*). Work with the library was performed in a P2 laboratory using SAGENE (South African Committee for Genetic Experimentation) guidelines.

2.3.1 Titration of the bacteriophage in the library.

Media were prepared as follows and sterilised by autoclaving:

NZCYM broth: (per litre)

10 g NZ-amine
5 g Bacto-yeast extract
5 g NaCl
1 g Casamino acids
2 g $MgSO_4$
(pH 7.5)

NZCYM bottom layer:

NZCYM broth pH 7.5
12 g agar / litre

NZCYM top layer:

NZCYM broth pH 7.5
6 g agarose / litre

Bacteriophage were grown as described in Maniatis *et al.* (1978). Dilutions of the library lysate were made into bacteriophage dilution buffer (10 mM Tris-HCL pH 7.0, 10 mM $MgCl_2$). A 0.5 ml aliquot of an overnight culture of the *E. coli* host strain was diluted into 3 ml NZCYM broth and grown at 37°C to OD_{600} = 0.6. 0.1 ml of diluted bacteriophage was then added to 0.1 ml *E. coli* and allowed to adsorb at 37°C for fifteen to thirty minutes, followed by addition of 6 ml molten (55°C) NZCYM containing 0.6% agarose (SeaKem). This top layer was then poured onto dry NZCYM plates containing 12% agar. Top agarose was used rather than agar since it peels off the plates less easily. The plates were incubated at 37°C overnight.

The optimum number of bacteriophage per plate required for screening the library was estimated from plates containing the maximum number of plaques that were non-overlapping.

2.3.2 Transfer of plaque DNA to nitrocellulose.

The protocol of Benton and Davis (1977) was followed. A number of plates containing the optimum number of bacteriophage were prepared. Individual bacteriophage colonies were stabbed, using sterile toothpicks, and re-inoculated onto *E. coli*-seeded agar plates in such a

way that regularly spaced arrays of fifty bacteriophage per plate were obtained. Plates were incubated at 37°C overnight and were then chilled at 4°C for at least one hour to allow the top agarose to harden.

A numbered nitrocellulose filter disc (Millipore HAWP) was placed carefully over each plate using blunt forceps and left for one minute. The filter and plate were marked asymmetrically with pencil so that they could be realigned after autoradiography. The filter was then laid, plaque side up, on a pad of blotting paper saturated with denaturing solution (0.5 M NaOH, 1.5 M Tris-HCL pH 7.0). It was left for two minutes and then dropped into a beaker containing approximately 200 ml neutralising solution (0.5 M Tris-HCL pH 7.0, 3 M NaCl). Many filters could be accumulated in this solution. The solution was changed once and the filters were then rinsed in 2 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M trisodium citrate), blotted dry and baked in a vacuum oven at 80°C for two hours.

2.3.3 Identification of Lambda Charon 4A recombinant bacteriophage containing unique sequence human DNA inserts.

The nitrocellulose discs containing transferred plaque DNA were hybridized to radiolabelled total human genomic DNA (see Section 2.6.1) and autoradiographed

for two to six hours (see Section 2.6.2). Plaques that did not appear to hybridize to total human genomic DNA were assumed to lack human repetitive DNA sequences. This was confirmed by repeating the Benton and Davis screening procedure (see Section 2.3.2) with putative plaques containing single-copy DNA, and rehybridizing the resultant filters to radiolabelled total human DNA.

2.3.4 Storage of recombinant bacteriophage

Plaques corresponding to regions of non-hybridization on the autoradiograph were assumed to contain bacteriophage with unique sequence human DNA inserts. Individual plaques were stabbed using a sterile Pasteur pipette, and resuspended in an Eppendorf tube containing 1.0 ml SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCL pH 7.5, 0.001% gelatin) and two drops of chloroform. Eppendorfs were stored at 4°C.

2.4 Preparation of human genomic DNA for analysis with DNA probes

2.4.1 Venous blood collection

Peripheral blood specimens were obtained from consenting individuals. Samples were collected by venipuncture into vacutainer tubes (Radem laboratory equipment (Pty) Ltd, Sandton, South Africa) containing

either the anti-coagulant ACD (for red blood cells, buffy coat and plasma) or EDTA (for buffy coat), or containing no anti-coagulant (for a clotted sample and serum). Sixty ml of blood was collected if possible; however, as little as five ml was sufficient for the extraction of approximately 50 µg DNA. Samples were centrifuged in order to separate the buffy coat, plasma/serum, and red blood cells. These were stored at -70°C, with the exception of the red cells, which were rapidly mixed with an equal volume of preserving fluid (60 mM Tripotassium citrate [$K_3C_6H_5O_7H_2O$]; 20 mM Sodium dihydrogen phosphate [$NaH_2PO_4.2H_2O$]; 20 mM Disodium hydrogen phosphate [Na_2HPO_4]; 400 ml Glycerol) and stored at -20°C. In the field, samples were either flown back to the laboratory within 48 hours of collection, or separated (using a portable petrol engine and generator-powered centrifuge if necessary) and temporarily stored in a portable liquid nitrogen container.

2.4.2 Extraction of high-molecular-weight DNA from leucocytes

Total genomic DNA was extracted from buffy coats according to the method of Michèle Ramsay (personal communication, 1984) (Method I), or the method of Sykes (1983) (Method II). The latter method was used more recently and found to be a more efficient means of

preparing good yields of quality DNA. This is attributed to the shorter preparation time required and therefore the less prolonged and harsh treatment of both cells and DNA.

Preparation of organic reagents

Liquid phenol was equilibrated to pH ≥ 7.5 by several extractions with an equal volume of buffer (0.1 M Tris-HCl pH 8.0). Small volumes of equilibrated phenol were freshly prepared, since the phenol was found to oxidise after several days, even after addition of the antioxidant 8-hydroxyquinolinol to a final concentration of 0.1%. Aliquots were stored at 4°C.

Chloroform and isoamyl alcohol were mixed 24:1 v/v and stored at room temperature.

Method I

The thawed buffy coat was resuspended in 10 ml STE (0.1 M NaCl, 0.01 M Tris-HCL pH 8.0, 1 mM EDTA) in a 100 ml conical flask. SDS was added to a final concentration of 0.5%, followed by proteinase K to a final concentration of 100 µg/ml. The flask was shaken either at 37°C overnight or at 55°C for two hours. Approximately one volume phenol was then added and the flask shaken slowly at room temperature for thirty

minutes. The solution was centrifuged for ten minutes at 2 000 *g* and the aqueous layer retained. The extraction was repeated using one volume of phenol:chloroform:isoamyl alcohol (25:24:1). 1 ml 3 M sodium acetate pH 5.6 was then added and mixed in well, followed by addition of 11 ml ice-cold isopropanol. DNA was spooled onto a sterile glass rod and redissolved in 5 ml TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA) overnight at room temperature.

The following day RNAse (Sigma) was added to a final concentration of 100 µg/ml and the sample incubated at 37°C for two hours. SDS was then added to a final concentration of 0.5%, as was proteinase K (Boehringer Mannheim) to a final concentration of 100 µg/ml. The sample was incubated for at least one further hour at 37°C. A phenol:chloroform:isoamyl alcohol extraction was performed as described above, followed by an extraction with one volume of chloroform:isoamyl alcohol (24:1 v/v). Each extraction required shaking for twenty minutes. The final aqueous layer was transferred to a clean conical flask, and a tenth volume of 3 M sodium acetate pH 5.6 was added and mixed in well. DNA was precipitated in a 1.1 volume of isopropanol, spooled onto a sterile glass rod, and redissolved in 500 µl - 1 ml TE buffer pH 8.0. The DNA was stored at 4°C.

DNA concentration was estimated by determining the absorbance at 260 nm, assuming that the $A^{1\%}_{1cm,260}$ of DNA is 200 (i.e. a 1 g/100 ml solution in a 1 cm lightpath has an absorbance at 260 nm of 200) (Old and Higgs, 1983). One OD_{260} was accepted as being equivalent to 50 µg of genomic DNA per ml.

Method II

The buffy coat, stored at -20°C with or without red cells, was thawed. An equal volume of 0.2% Triton-X (Sigma); 0.9% NaCl was added and well mixed, and the tube was centrifuged at 2 000 *g* for fifteen minutes. The supernatant fluid was discarded and the soft pellet was resuspended in 30 ml 0.2% Triton-X, 0.9% NaCl by vigorous shaking. After centrifugation at 2 000 *g* for fifteen minutes a pink-white pellet was obtained; however, if the pellet was still very red the saline wash was repeated. Lysing buffer (7 M Urea, 0.3 M NaCl, 10 mM EDTA, 10 mM Tris-HCL pH 7.5) was added, a few drops at a time to a total volume of 10 ml, and the pellet was worked into solution using a wide diameter glass rod. SDS was then added to a final concentration of 2%, and the mixture was incubated at 37°C for ten minutes.

Phenol extractions were performed: two with (5 ml chloroform:isoamyl alcohol and 10 ml phenol) followed

by one with 5 ml chloroform:isoamyl alcohol only. For each extraction the aqueous and organic phases were shaken vigorously, and the aqueous layer was retained after separation by centrifugation at 2 000 *g* for ten minutes. DNA was precipitated in 1.1 volumes of ice-cold isopropanol, spooled onto a sterile glass rod, redissolved in 1.0 - 1.5 ml TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA), and stored at 4°C. DNA concentration was measured as described for method I.

2.4.3 Restriction endonuclease digestion of genomic DNA

The following type-II restriction enzymes (Old and Primrose, 1980) were used in this study: *Ava*II (Amersham); *Bam*HI (Anglian); *Bgl*II (Anglian); *Eco*RI (Anglian); *Hind*II (Boehringer Mannheim); *Hind*III (Anglian); *Mbo*I (BRL); *Msp*I (BRL); *Pst*I (Amersham); *Pvu*II (Amersham); *Rsa*I (Boehringer Mannheim); *Sst*I (BRL); *Stu*I (Boehringer Mannheim); *Taq*I (Boehringer Mannheim). Their respective nucleotide recognition sites are shown in Table 2.1.

Incubation mixtures with final volumes of 50 - 100 µl were prepared. Digests contained the following: 10 µg of human DNA or 0.5 - 1.0 µg of phage DNA; an incubation buffer prepared according to manufacturer's instructions; 2.5 units of restriction enzyme per µg of

Table 2.1 Restriction enzymes used in the study

Name of enzyme	Recognition [a] sequence	N° of bp recognised
TaqI	T/CGA	4
MspI	$\overset{+}{C}/\overset{\circ}{C}GG$	4
BglII	$A/G\overset{\circ}{A}TC\overset{+}{T}$	6
HincII	$GT\begin{matrix}(T)(A)\\(C)(G)\end{matrix}AC$	6
SstI	GAGCT/C	6
StuI	AGG/CCT	6
PstI	$CTGC\overset{+}{A}/G$	6
AvaII	$G/G\begin{matrix}(A)\\(T)\end{matrix}C\overset{+}{C}$	5
BamHI	$G/GATC\overset{+\circ}{C}$	6
EcoRI	$G/\overset{+}{A}ATT\overset{+}{C}$	6
HindIII	$\overset{+}{A}/AG\overset{+}{C}TT$	6
PvuII	CAG/CTG	6
MboI	$G\overset{+}{A}T\overset{\circ}{C}$	4
RsaI	GT/AC	4

[a] From Kessler et al(1985)

/ Represents the cleavage site

+ Indicates the inhibition by methylation

m Indicates the requirement of methylation for enzyme activity

° Indicates that methylation has no effect on enzyme activity

DNA (where one unit is defined as that enzyme activity which completely digests 1 µg of lambda DNA in one hour at the appropriate temperature under optimal assay conditions); 4 mM spermidine trihydrochloride (Sigma); and sterile distilled water to make up the volume. The addition of the polyamine, spermidine, was found to reproducibly improve the success of reactions, probably by modifying the DNA conformation to a condensed, torus-like structure (Marx and Ruben, 1983) more easily able to interact with moieties in the reaction mixture.

The reaction mixture was incubated at the recommended temperature and for the time required for the enzyme to digest the DNA to completion, usually 37°C or 65°C for four to six hours or overnight. Optimal conditions often required empirical determination. In general, enzyme activity is lost after five hours of incubation (Crouse and Amorese, 1986), and some enzymes, notably *Msp*I, performed better in short incubation times. After incubation, any condensate was collected by centrifugation in an Eppendorf microfuge.

Incubation mixtures with volumes greater than 60 µl required precipitation and resuspension in a smaller volume. An ethanol precipitation was usually performed as follows: a tenth volume of 3 M sodium acetate pH 5.6 was added to the DNA solution and mixed well; this was followed by the addition of two volumes of ice cold 95%

ethanol; and tubes were then placed at -70°C for twenty minutes or at -20°C overnight. Tubes were then centrifuged at 2 000 *g* for thirty minutes at 4°C and the supernatant discarded. The DNA pellet was washed with 70% ethanol twice, which entailed centrifuging the tubes after addition of ethanol, and discarding the supernatant. The pellet was then placed in a vacuum oven or left at room temperature for at least thirty minutes until the ethanol had evaporated. It was redissolved in 50 - 100 µl TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA), depending on the size of the pellet.

A 0.1 volume aliquot was removed from each incubation mixture and electrophoresed in order to determine whether digestion was complete. Complete digestion was assumed if the DNA in the gel was an even smear, with no strong high-molecular-weight band present. After confirmation of digestion a 0.1 volume of a 5 x loading buffer (50% sucrose, 50 mM EDTA, 2% ficoll, 0.1% bromophenol blue) was added. This stopped the reaction and was required for the gel electrophoresis, both as a dense loading medium and as a tracking dye.

2.4.4 Agarose gel electrophoresis of DNA restriction fragments

0.6% - 1.2% agarose horizontal slab gels were prepared by adding agarose powder (HGT, SeaKem) to TEA buffer

(0.04 M Tris-acetate; 0.02 M EDTA) and boiling until completely dissolved. The solution was cooled to approximately 60°C and ethidium bromide was added to a final concentration of 0.5 µg/ml. It was then poured into a gel mould with a comb in place and allowed to set at room temperature. The gel trough had been previously bleached for at least thirty minutes and then washed thoroughly in distilled water. This prevented contamination by residual bacteriophage or plasmid DNA.

DNA samples containing loading buffer and one to two different molecular weight markers were loaded into the gel slots. Markers were prepared by digestion of lambda bacteriophage (Boehringer Mannheim) with the endonucleases *Eco*RI, and *Hind*III, singly or in combination. Approximately 20 ng marker DNA and 10 µg sonicated salmon sperm DNA (Sigma) were electrophoresed in the marker lane.

Gels were submerged in TEA buffer and a current applied. Trial gels for determination of complete digestion were electrophoresed at 80 - 100 mA for one to two hours; gels for separating DNA restriction fragments prior to Southern transfer were electrophoresed at 50 - 80 mA for one to three days, depending on the fragment sizes under investigation. Separation of large fragments was improved by prolonged electro-

phoresis. After electrophoresis was completed, as determined by the migration of the bromophenol blue of the loading buffer to the end of the gel, the gel was placed on a UV transilluminator (Chromato-Vue, model O-63) and photographed on Polaroid type 665 (positive/negative) or 667 (positive) film with a yellow filter. An example of human DNA samples electrophoresed in agarose is shown in Figure 2.2.

2.4.5 Transfer of DNA fragments in agarose onto nitrocellulose or nylon membranes

2.4.5.1 Comparison of nylon and nitrocellulose transfer membranes

The procedure developed by Southern (1975) for transferring DNA fragments *in situ* from agarose gels to nitrocellulose membranes, followed by hybridization with a radioactive complementary DNA probe, remains basically unmodified. Recently, however, a number of alternative nylon-based membranes have become commercially available. The author has compared several of these membranes with nitrocellulose (Schleicher and Schüll), namely: Biodyne (PALL); Hybond-N (Amersham); Gene-Screen* (New England Nuclear); and Zeta-probe (Chemlab). The comparison was based on the following criteria: ease of use, efficiency of DNA transfer, efficiency of probe

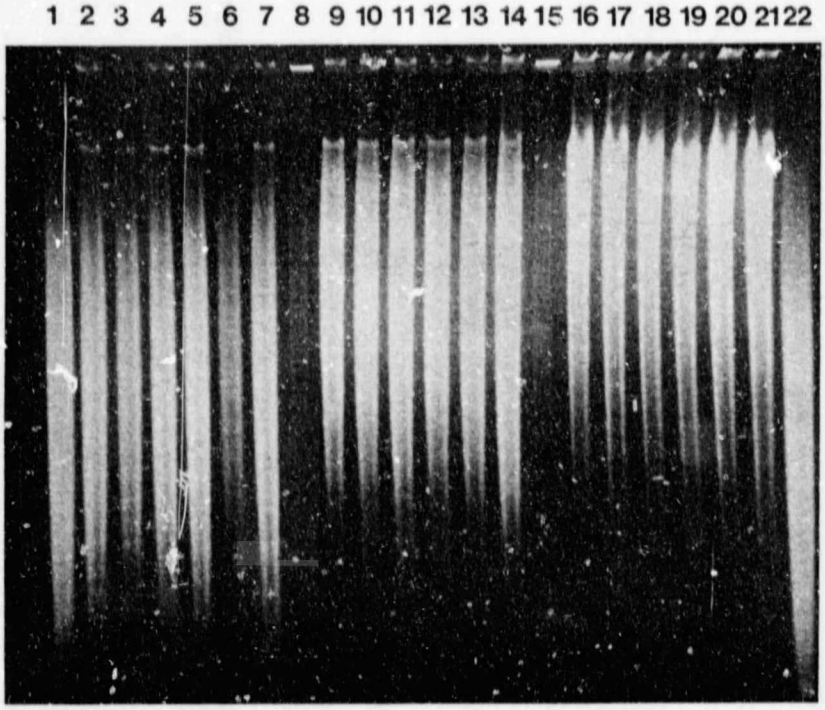

Figure 2.2 Example of human genomic DNA digestions electrophoresed in 0.7% agarose prior to Southern transfer. The gel was stained with ethidium bromide and visualised under ultraviolet light. Restriction enzymes used for digesting the DNAs were AvaII (lanes 2 - 7), HincII (lanes 9 - 14) and BamHI (lanes 16 - 21). Lanes 1, 8, 15 and 22 contain Lambda molecular-weight markers.

hybridization, and lack of background hybridization. Duplicate digestions were electrophoresed in the same gel and hybridized to different membranes. Transfer solutions recommended by the manufacturers were used and DNA was efficiently transferred to each membrane. The Biodyne protocol was easiest to follow, followed by Zeta-probe, and the clearest hybridization results were obtained with Biodyne again followed by Zeta-probe. The experiment did not take into account possible variations in either batch quality or technical error, since it was not repeated. However, for the purposes of the laboratory, immediate ease of use without undue experimental manipulation was required, and the laboratory adopted Biodyne (PALL) as the membrane of choice, with some limited use of Zeta-probe (Chemlab).

Nylon membranes have proved to be more pliable than nitrocellulose, easier to handle, and virtually indestructible, permitting multiple reprobing of the membranes. The procedures reported here are adapted from the manufacturer's recommendations.

2.4.5.2 Southern transfer

In an early protocol the DNA in the agarose gel was exposed to UV light for five to ten minutes in order to facilitate transfer by creating smaller fragments (Wahl *et al.*, 1979). UV exposure was later minimised, how-

ever, since it was found to actually inhibit transfer (Reed and Mann, 1985; and the author's experience). For transfer to all membranes the gel was submerged in 250 ml denaturing solution (0.5 M Tris-HCL, 3 M NaOH) for thirty minutes, with gentle shaking, and then rinsed in distilled water. This was followed by immersion in 250 ml portion(s) of neutralising solution for thirty minutes at a time: for nitrocellulose and for Zeta-probe in three portions of (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl); and for Biodyne in one portion of 1 M sodium acetate pH 5.6. The gel was then rinsed in water and soaked in 10 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M trisodium citrate pH 7.0) for at least ten minutes.

Transfer took place on a glass plate supported on a trough filled with 10 x SSC. A piece of Whatman 3MM filter paper was soaked in 10 x SSC and draped over the glass plate with its ends in the SSC solution. The gel was placed on the wet filter paper, avoiding air bubbles, and the paper around the gel was covered with a layer of waterproof paper. The transfer membrane was cut to the size of the gel, wet by floatation on 2 x SSC and carefully placed on the gel. A sterile pipette was used to roll away any air bubbles or unevenness. Two pieces of Whatman 3MM paper, cut to gel size and soaked in 2 x SSC, were then placed on the membrane. Dry absorbent paper was placed onto the filter paper

and compressed with a glass plate and a suitable weight, usually a small telephone directory.

Transfer of the DNA fragments in the gel to the membrane support took place overnight at room temperature. The well positions were then marked and the membrane blotted dry. DNA was fixed to the membrane by baking at 80°C for one to three hours in a vacuum oven. The dry membrane was stored in a sealed plastic bag at room temperature prior to hybridization (see Section 2.6.2.).

2.5 Preparation of bacteriophage and plasmid DNA for use as DNA probes

DNA was isolated from large-scale preparations of bacteriophage or plasmid vectors. DNA from phage required analysis in order to determine whether an insert was present or not, before it could be used as a hybridization probe.

2.5.1 Purification of DNA from large-scale preparations of bacteriophage containing putative single-copy DNA inserts

A modification of the method of Bowcock (1984) was used. Media were prepared as for Section 2.3.1, except that agar was substituted for agarose in the top layer.

The preparation of high titres of phage and the purification and concentration of phage DNA is described below.

Preparation of high titres of bacteriophage

Plating cells were prepared from an overnight culture of *E. coli* LE392 by adding 0.5 ml of the overnight culture to 3 ml NZCYM broth. 0.1 ml aliquots of the plating cells were added to 0.1 ml of serial dilutions in phage buffer (10 mM Tris-HCL pH 7.0, 10 mM $MgCl_2$) of the bacteriophage suspended in SM buffer (see Section 2.3.4.), and allowed to adsorb for at least fifteen minutes at 37°C. 5.0 ml NZCYM top agar (melted and cooled to 55°C) was added to each dilution and these were poured onto dry NZCYM agar plates. After allowing the plates to dry for fifteen minutes, they were incubated, inverted, at 37°C overnight. The following day forty fresh plaques of approximately the same size from plates with isolated plaque colonies were picked into 3.0 ml of fresh LE392 plating cells and incubated at 37°C for fifteen minutes to one hour. Approximately 70 ml of NZCYM top agar was added to each sample and then divided amongst ten NZCYM plates (8 cm diameter), working sterilely. Plates were incubated at 37°C until lysis was confluent (approximately four to six hours).

Once confluent lysis was evident, with a typical lacy pattern on each plate, plates were incubated at 4°C for at least thirty minutes. 7.5 ml cold phage buffer was added to each plate and plates were incubated at 4°C overnight. The following day the buffer was pipetted off the plates and 2 ml chloroform added for complete lysis. The bacteriophage lysate was titrated, and a titre of 10^{10} pfu/ml was found to represent sufficient bacteriophage, containing an amount that could be visualised on a CsCl gradient.

At this stage it was possible to add DNAse (Sigma) and RNAse (Sigma) to a final concentration of 100 µg/ml to the lysate, and incubate at 37°C for two hours. However, if this step was omitted then RNAse to the same final concentration was added to the DNA before the phenol extraction stage (see below).

The supernatant fluid constituting phage lysate was centrifuged at 9 000 *g* for ten minutes at 4°C. The pellet containing cell debris was discarded, and the SNF was centrifuged at 12 500 *g* for two hours and thirty minutes at 4°C. A small green translucent phage pellet was obtained, which was resuspended in 1.5 ml cold phage buffer, and left at 4°C overnight or up to three days.

Purification and concentration of bacteriophage DNA

A CsCl step gradient was prepared with three densities of CsCl in phage dilution buffer: 1.3; 1.5; and 1.7 g/ml. The phage solution was layered onto the gradient, and tubes were filled with mineral oil. A Beckman SW40Ti rotor was used to centrifuge the tubes at 15 300 *g* for two hours at 20°C. Phage particles were visible as a distinct blue band at a density of approximately 1.5 g/ml. The band was removed by piercing the side of the tube using an 18G needle. A 0.5 to 1.5 ml volume was usually collected. This solution was dialysed against phage dialysis buffer (50 mM Tris-HCL pH 8.0, 10 mM NaCl, 10 mM $MgCl_2$) for at least one hour to remove the CsCl.

After dialysis, the phage solution was extracted with an equal volume of equilibrated phenol (see Section 2.4.2): the organic and aqueous phases were mixed gently, and the organic phase containing protein was discarded. The extraction was repeated four times.

After the final phenol extraction, the aqueous layer containing phage DNA was removed and the volume measured. An ethanol precipitation step was performed as described in Section 2.4.3. Phage DNA was stored at 4°C.

5 µl of the DNA solution was electrophoresed in a 0.6% agarose gel in order to determine whether the DNA was of high molecular weight. If the DNA was not degraded, a single intense band was visible close to the gel slots. Concentration was estimated as described (see Section 2.4.2.)

2.5.2 Distinguishing of recombinant from non-recombinant bacteriophage

In order to determine whether the putative single-copy bacteriophage (identified by their lack of hybridization to total human genomic DNA) were lacking in human repetitive DNA or did not contain a human insert at all, 2 - 5 µg of DNA extracted from such phage was digested with the restriction endonuclease *Eco*RI. This enzyme has a recognition site at the junction between the lambda Charon 4A arms and the inserted human DNA, and could also have recognition sites within the inserted DNA. After digestion, the fragments were subjected to agarose gel electrophoresis. Non-recombinants could be identified as having five fragments, of 30.7, 19.8, 10.9, 7.8, and 6.8 kb. The 19.8 and 10.9 kb fragments represented the lambda Charon 4A arms, and the 30.7 kb fragment occurred if the *cos* sites in the arms annealed. Recombinants lacked the 7.8 and 6.8 kb fragments, but

contained additional fragments, representing the human DNA insert.

The DNA in the gel was transferred to nitrocellulose in order to detect human repetitive DNA in any phage that were not excluded in the initial screening procedure. Repetitive DNA was detected by hybridizing the nitrocellulose filters with radiolabelled total human genomic DNA; phage with human DNA inserts that still failed to hybridize could be used as single-copy probes.

2.5.3 Purification of plasmid DNA from large-scale preparations of amplified plasmid

The protocol used was that of Maniatis *et al.* (1982). Transformed *E. coli* HB101 hosts containing the plasmids pAW101 and $^{A}\gamma$-globin (Wainscoat *et al.*, 1986b) were gifts from R. White and J. Wainscoat. The plasmids were amplified in rich medium, their bacterial hosts harvested and lysed, and the plasmid DNA extracted.

Amplification in rich medium

Media were prepared as follows and sterilised by autoclaving:

LB broth: (per litre)

10 g Bacto-tryptone

5 g Bacto-yeast extract

10 g NaCl pH 7.5

LB agar:

LB broth pH7.5

12 g agar/litre

10 ml LB broth containing 100 µg/ml ampicillin (Merck) was inoculated with a single *E. coli* colony containing the required plasmid. Both plasmids conferred ampicillin-resistance to their hosts. The flask was incubated at 37°C overnight with vigorous shaking. 0.1 ml of the overnight culture was used to inoculate 40 - 50 ml of ampicillin-containing LB broth, and the flask was incubated at 37°C with vigorous shaking until the culture reached late log phase (OD_{600} = 0.6). A 2-litre flask with 500 ml of ampicillin-containing LB broth, prewarmed to 37°C, was then inoculated with 25 ml of the late log culture. This was incubated at 37°C and shaken until the OD_{600} = 0.4. Chloramphenicol (Sigma) was added to a final concentration of 170 µg/ml, and the culture was incubated at 37°C with vigorous shaking for a further twelve to sixteen hours.

Harvesting and lysis of bacteria

The bacterial cells were harvested by centrifugation at 4 000 *g* for ten minutes at 4°C. The bacterial pellet was then washed in 100 ml cold STE (0.1 M NaCl, 10 mM tris-HCl pH 7.8, 1 mM EDTA), and resuspended in 8 ml of

a cold solution of 10% sucrose in 50 mM Tris-HCl pH 8.0. 2 ml of a freshly-made solution of lysozyme (Sigma, 10 mg/ml in 0.25 M Tris-HCl pH 8.0) was added to the solution, followed by 8 ml of 0.25 M EDTA. The tube was inverted several times and placed on ice for ten minutes. 2% SDS was added, the bacterial suspension mixed quickly but gently, and 6.0 ml 5 M NaCl was then mixed into solution.

The solution was placed on ice for at least one hour. It was then centrifuged at 13 000 *g* in a Beckman JA20 rotor at 4°C for one hour to pellet high-molecular-weight DNA and bacterial debris. The supernatant was retained and extracted twice with phenol:chloroform and once with chloroform. Two volumes of ethanol were added to the aqueous phase, mixed and allowed to stand at -20°C for one to two hours. The nucleic acids were recovered by centrifugation at 1 500 *g* for fifteen minutes at 4°C, and the pellet was washed with 70% ethanol at room temperature. Excess ethanol was allowed to evaporate and the DNA was dissolved in 8 ml TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA).

Purification and concentration of plasmid DNA

CsCl was added to the DNA solution to a final concentration of 0.95 g/ml, and mixed gently. 0.1 ml of 5 mg/ml ethidium bromide was then added and mixed in

well. The final density of the solution was adjusted to 1.55 g/ml (η = 1.3860). The solution was transferred to a tube suitable for centrifugation in a Beckman Type-65 Ti rotor and the remainder of the tube was filled with mineral oil. It was centrifuged at 13 200 *g* for two to three days at 20°C. The DNA was viewed with long wavelength ultraviolet light and the lower plasmid band was collected with a syringe from above. The ethidium bromide was removed by several isoamyl alcohol extractions, and the aqueous phase was dialysed against several changes of TE for two to three days. DNA concentration was estimated by determining its absorbance at 260 nm.

2.6 Analysis of human DNA fragments using DNA probes

DNA hybridization probes were prepared by radiolabelling with ^{32}-P, and probes were then used to detect homologous DNA sequences immobilised on Southern transfer membranes. The membranes could be re-used after removal of previously-bound probe.

2.6.1 Radiolabelling of the DNA probe

DNA was prepared for use as a probe by incorporating radioactive nucleotides into the DNA in a nick translation reaction. Unincorporated nucleotides were then removed by fractionation in Sephadex.

Nick translation

A modification of the protocol of Rigby (1977) was followed. Nick translations were carried out in 1.5 ml Eppendorf tubes in a total volume of 25 - 100 µl. Distilled water (if required to make up the volume) was pipetted into the tube, followed by: 0.5 - 1.0 µg of the probe DNA; the appropriate volume of a nick translation buffer stock solution (10 x buffer = 500 mM Tris-HCl pH 7.5; 50 mM $MgCl_2$; 100 mM β-mercaptoethanol; 0.2 mM dGTP; 0.2 mM dATP; 0.2 mM dTTP); 50 µCi (α-^{32}P)-dCTP (Amersham); 5 units of commercial Polymerase I (Boehringer Mannheim); and finally 1 µl of a 2 x 10^{-5} mg/ml stock solution of DNAse I (Sigma). A nick translation kit (Amersham) has also been used successfully. The solution was mixed gently and incubated at 15°C for thirty to ninety minutes. The percentage incorporation was calculated at approximately half-hourly intervals, and the reaction mixture was placed on ice when maximum incorporation was reached. At this stage it was found possible to freeze the reaction mixture.

Incorporation of ^{32}-dCTP was estimated as follows: 0.5 µl of the reaction mixture was spotted onto a Whatman 2.5 mm GF/C filter. This was counted on a scintillation meter type 540 (total counts). The filter was then washed once with 10% TCA + 0.2 M pyrophosphate and

three times with 5% TCA, and counted (acid precipitable counts). Percentage incorporation could be calculated as:

$$\frac{\text{Acid precipitable counts}}{\text{Total counts}} \times 100$$

Percentage incorporation of more than 40% was usually found to be sufficient for efficient hybridization of the probe to target DNA.

Fractionation

To each nick translated mixture (unthawed if frozen), 0.5 ml TE buffer containing 100 µg/ml sonicated and denatured salmon sperm DNA (Sigma) was added, followed by 0,5 ml Tris-equilibrated phenol containing 0.1% 8-hydroxyquinol. Tubes were shaken vigorously and centrifuged in an Eppendorf microfuge. The aqueous phase was removed and mixed with a tiny amount of Orange G dye (Sigma), which migrates just behind the unincorporated nucleotides. Labelled DNA was then separated from the free nucleotides by chromatography on a small column of Sephadex G-50 (fine) (Pharmacia). The Sephadex G-50 had been previously swelled in TE (5% weight/volume) for at least three hours. The DNA was eluted with TE and collected in fractions, which were kept on ice. The excluded peak containing the labelled DNA was pooled and denatured in a 90 - 100°C waterbath for ten minutes, and then placed immediately on ice.

If not used within a few hours, the labelled and fractionated DNA was frozen at -20°C for up to four days until required.

10 µl of the labelled probe was placed in a scintillation vial and specific activity (Cherenkov counts) was measured in a β-counter (LKB Wallack 1217 Rackbeta liquid scintillation counter). Between 1 x 10^7 and 1 x 10^8 cpm/µg DNA was usually obtained.

2.6.2 Hybridization of the radiolabelled probe to nitrocellulose or nylon membranes containing human DNA fragments, and autoradiography

Prior to hybridization, Zeta-probe membranes required washing at 65°C for one or more hours in a preheated solution of 0.1 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0); 0.5% SDS.

Baked membranes, wet in 2 x SSC, and pretreated Zeta-probe membranes, were sealed in a plastic bag with 10 - 15 ml prehybridization solution (50% formamide; 3 x SSC; 5 x Denhardt's solution (1 x = 0.02% PVP, 0.02% Ficoll, 0.02% BSA (Sigma)); 250 µg/ml sonicated and denatured salmon sperm DNA (Sigma); 10 µg/ml polyadenylic acid (Boehringer Mannheim); 0.05 M Hepes pH 7.0; 0.1% SDS). Up to five membranes could be sealed in one bag. Care was taken to exclude air

bubbles from the bag, and membranes were shaken gently at 42°C for at least six hours.

Pre-hybridization solution was extruded from the plastic bag containing the membrane(s) and 8-15 ml hybridization solution (prehybridization solution containing 10% dextran sulphate (Sigma)) was added, as was the heat-denatured probe DNA. Molecular weight markers were detected by hybridization to radiolabelled lambda DNA, also added to the hybridization mixture. The bag was then re-sealed, avoiding trapping any air bubbles. Membranes were incubated at 42°C for 16 - 24 hours, with gentle shaking.

The hybridization mixture containing the radiolabelled probe could be re-used, after denaturing the probe DNA by heating to 70°C.

After hybridization, the membranes were rinsed and washed in several changes of 2 x SSC at room temperature for a total of thirty minutes. Nitrocellulose filters were then washed at 55°C in 0.1 x SSC, 0.1% SDS for one hour. Nylon membranes were washed at 42°C in 0.1 x SSC, 0.1% SDS for thirty minutes, followed by a wash at 65°C in fresh prewarmed 0.1 x SSC; 0.1% SDS. All membranes were given a final rinse in 0.1 x SSC at room temperature, and then sealed individually in plastic bags while damp.

The membranes were autoradiographed using Trimax 3 M type XD or Kodak XAR-5 X-ray film. The film and the filter were placed in a cassette, between two Trimax 3 M or Cronex Hi-plus Xtra life (DuPont) intensifying screens, and the film was exposed at -70°C for one to fourteen days. The film was then developed in Kodak GBX developer for three to five minutes, rinsed for thirty seconds in water, fixed in Kodak Rapid Fixer for five minutes, washed in water for fifteen minutes and allowed to dry.

If excess background hybridization was evident, the filter was given a stringent wash at 65°C or 70°C in 0.05% SDS; 0.05 x SSC, and re-autoradiographed.

2.6.3 Re-use of hybridization membrane

After autoradiography, the membranes could be re-used: up to four times with a nitrocellulose filter; and up to ten or fifteen times with a nylon membrane. Multiple re-probing first required removal of the previously bound probe. This was achieved for nitrocellulose by placing the filter in denaturing solution for five minutes, rinsing with distilled water, and neutralising in two changes of solution for ten minutes. Nylon membranes were incubated in 0.4 M NaOH at 42°C for thirty minutes, followed by a wash at 65°C

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