Volume 14 Number 18 1986 Nucleic Acids Research

Genomic analysis II: isolation of high molecular weight heteroduplex DNA following differential methylase protection and Formamide-PERT hybridization

Nancy J.Casna, David F.Novack, Ming-Ta Hsu+ and John P.Ford

Lifecodes Corporation, 4 Westchester Plaza, Elmsford, NY 10523 and +Mount Sinai School of Medicine, City University of New York, New York, NY 10029, USA

Received 29 May 1986; Revised and Accepted 25 August 1986

ABSTRACT

Understanding the nature of DNA sequence differences among individuals is important to the understanding of fundamental questions in biology. To analyze such differences in complex genomes new approaches must be developed. We report two new techniques which aid in this effort. First, we have developed a modification of the Phenol Emulsion Reassociation Technique (PERT) that allows hybridization of long (20 kb and longer) single copy heteroduplex DNA fragments from human genomic DNAs. Secondly, by using a differential methylase protection technique we have shown that double methylase resistant heteroduplex DNA molecules can be size fractionated away from reannealed single methylase resistant homoduplex DNA molecules. These methods will be useful in obtaining DNA from chromosomal subregions linked to the inheritance of a specific trait or condition as described in the preceding paper (1) and could also be used to create a map of the chromosomal subregion which includes the gene for the trait.

INTRODUCTION

Defining DNA sequence divergence is important in understanding oncogenesis and genetics (2). The analysis of sequence divergence in complex genomes such as that of the human requires the development of new approaches. First, there is the need for techniques capable of detecting minimal DNA base sequence changes. Our laboratory and others have developed techniques which identify unpaired bases in DNA and RNA molecules that result from sequence differences as small as a single point mutation (3-6). In order to assess mutation frequency and distribution in a statistically meaningful manner, one must analyze long heteroduplex DNA fragments. The reassociation of high molecular weight DNA from a genome of high complexity, as well as the occurrence of DNA self-reannealing rather than heteroduplex formation, each present separate technical obstacles

© IRL Press Limited, Oxford, England.

which must be dealt with in order to approach the problem of sequence divergence in the human genome.

A technique for rapid reassociation of genomic DNA (PERT) which increases the hybridization rate by several orders of magnitude over aqueous hybridization conditions has been previously described (7). Co_t analysis of human genomic DNA with PERT suggested that unique sequence DNA could be reannealed in about 16 h at a concentration of 0.5 ug/ml. Using the technique as described by Kohne et al. (7) however, we have consistently found that the hybridized DNA following the PERT reaction is shortened to < 4 kb. Recently, using the PERT technique, Kunkel et al. cloned specific fragments of the X-chromosome (8). The average size of the cloned fragments was relatively small (< 200 bp) and therefore was not sensitive to the loss of DNA fragment length. We report a modification of PERT (Formamide-PERT, F-PERT) that allows accelerated reassociation of high molecular weight human DNA molecules, in which single copy sequences can be detected.

To generate heteroduplex DNA molecules, the DNA of two individuals is mixed in equal amounts, denatured and hybridized. During the hybridization both reannealed homoduplex and heteroduplex DNA molecules are formed. Since heteroduplex and homoduplex DNA molecules are equally represented in the reassociated population, a method must be developed to fractionate heteroduplex DNA away from reannealed homoduplex DNA.

Gruenbaum et al. (9) have reported that after methylation at the 5 position of cytosine of the target sequence in only one strand (hemi-methylated) DNA was resistant to restriction endonuclease digestion by 12 out of 17 enzymes tested with cytosine in the recognition site. In addition Gelinas et al. (10) showed that the restriction enzyme Mbo I only cleaves when adenines of the target sequence in both strands are unmethylated. We reasoned that heteroduplex DNA molecules in which each strand was methylated at a different site would be resistant to cleavage by both restriction endonucleases sensitive to hemi-methylation at those sites. Reannealed homoduplexes however, would be methylated in both strands at the same site and would thus be resistant to cleavage by only one of the two restriction enzymes.

We describe a method based on this principle for the fractionation by size, of heteroduplex DNA molecules from reannealed homoduplex DNA molecules.

These techniques for purifying long heteroduplex molecules will be useful in obtaining DNA from chromosomal subregions linked to the locus for a specific trait or condition. DNA fragments from a single chromosomal subregion could be the basis of a test for the inheritance of the trait. The DNA fragments, as a set, would also be useful in the creation of a map of the chromosomal subregion within which the gene for the trait is located (11). The rationale for such an approach is explained in the preceding paper (1).

MATERIALS AND METHODS

Restriction endonucleases and methylases were from Bethesda Research Laboratories or New England Biolabs and were used according to the manufacturer's recommendation. [α- ^{32}P] dATP (3000 Ci/mmole) and [γ - ^{32}P] ATP (> 3000 Ci/mmole) were obtained from Amersham International. Lambda DNA free of N^6-methyladenine was from Promega Biotec. Klenow fragment of DNA polymerase I, polynucleotide kinase, calf-intestine alkaline phosphatase and S1 nuclease were from Boehringer Mannheim Biochemicals. Phenol and formamide were from International Biotechnologies Inc. Phenol was redistilled and stored under nitrogen at -20°C as a 90% solution (with deionized water). Formamide was crystallized twice and also stored frozen. 3 M sodium thiocyanate (Kodak) was filter sterilized and stored at -70°C. Agarose gel electrophoresis in non-denaturing gels was carried out using slab gels of 0.6% - 1.2% agarose (containing 0.5 ug/ml ethidium bromide) in a buffer of 40 mM Tris (pH 8.3), 5 mM sodium acetate, 1 mM EDTA. Denaturing gel conditions were as described by McDonnell *et al*. (12). Bands were visualized by exposure to long-wave UV light or by autoradiography.

F-PERT reaction conditions: To insure that the integrity of the starting DNA for the F-PERT reaction was > 30 kb, human genomic DNA was prepared as described (13), spooled onto a glass rod and resuspended overnight in TE (10 mM Tris pH 7.9, 1 mM EDTA). The DNA was digested with the specified restriction

enzyme, phenol extracted and resuspended in TE. The digested DNA was denatured by adjusting the solution to 0.3 M with 5 M sodium hydroxide. After 15-20 min at room temperature, the solution was neutralized by the addition of 3 M MOPS to a final concentration of 0.4 M. The pH of the denatured and neutralized solutions was routinely monitored by spotting 1 ul onto pH paper. The denatured solution was pH >13, the neutralized solution pH 7-8. For hybridization a solution of the denatured DNA (1-100 ug/ml) was made 2 M sodium thiocyanate, 10 mM Tris (pH 7.9), 0.1 mM EDTA, 8% formamide. Lastly, 90% phenol was added until two phases formed (25-30% final concentration). The solution, in a 13 ml Sarstedt polypropylene tube, was gassed with nitrogen and shaken at room temperature at a setting of 3.5 on a Labline multi-wrist shaker for 20-24 h. This setting was chosen because it was the minimum setting which maintained the emulsion with the tubes held at an angle of about 30° to the horizontal. The aqueous phase was recovered by two extractions with an equal volume of chloroform. The DNA was precipitated by additon of 10 volumes of 100% ethanol in a 35 ml Sarstedt polypropylene tube. After 30 min at $-70^{\circ}C$, the DNA was recovered by centrifugation at 10,000 rpm for one h at $4^{\circ}C$ in an SS-34 rotor. For convenience, the pellet was resuspended in 500 ul TE and precipitated with ethanol (in 0.3 M sodium acetate pH 5.1) in the routine manner. The pellet was resuspended in TE and either run directly on a gel or further digested with enzymes. DNA was treated with nuclease S1 (100 units/ug) in 200 mM sodium chloride, 50 mM sodium acetate (pH 5.0), 2 mM zinc chloride at $16^{\circ}C$ for 30 min and analyzed by agarose gel electrophoresis.

Southern hybridization: Following electrophoresis in an agarose gel the DNA was denatured and transferred to a nylon membrane (Zetapore, from AMF Cuno) by the method of Southern (14) with the modifications recommended by the vendor. The probe was labelled by nick translation (15). Hybridization was in 10 ml 5X SSPE (1X SSPE = 150 mM sodium chloride, 10 mM sodium phosphate, 1mM EDTA, pH 7.4), 1% SDS, 10% dextran sulphate, 500 ug/ml heparin with 25 X 10^{6} cpm of probe at $65^{\circ}C$ for 18 h. The blot was washed for 15 min at $65^{\circ}C$ four times each with 2X SSPE, 1% SDS; 0.4X SSPE, 1% SDS and 0.1X SSPE, 1% SDS. The hybridized

bands were visualized by autoradiography at -70°C with Dupont Cronex Quanta III intensifying screens.

Electron Microscopy: DNA was prepared for electron microscopy by the formamide technique of Davis, et al. (16), stained with 0.05 M uranyl acetate in 0.05 M HCl and examined using a Phillips 201 electron microscope.

Methylation: Lambda DNA free of N^6-methyladenine was cleaved with Hind III, extracted with an equal volume of phenol, extracted with an equal volume of chloroform, precipitated with ethanol (in 0.3 M sodium acetate) and resuspended in TE to a concentration of 1 ug/ml. The digested DNA was methylated with Alu or Dam methylase in 50 mM Tris pH 7.5, 10 mM EDTA, 5 mM 2-mercaptoethanol, 80 uM S-adenosyl methionine (SAM) with two units of enzyme per ug of DNA for one h at 37°C. An additional equal amount of SAM was added to the tube and the incubation was continued for an additional h at 37°C. The completeness of methylation was checked on an agarose gel following digestion with Alu I or Mbo I. The methylated DNAs were mixed in a 1:1 ratio at a DNA concentration of 0.05-0.1 ug/ul and denatured by the addition of 5 M sodium hydroxide to a final concentration of 200 mM with incubation at room temperature for 10 min. The solution was neutralized to pH 7.5 with 3 M MOPS (2.5 times the volume of 5 M NaOH) and hybridized at 42°C for one to two h. The DNA was precipitated with ethanol, resuspended in TE and digested with Alu I and/or Mbo I. For the experiment utilizing labelled DNA, 1.5 ug of the Alu-methylated Hind III-digested lambda DNA in 1X CIP buffer (17) was treated with 12.5 units calf intestine alkaline phosphatase in a volume of 25 ul for 30 min at 37°C. The solution was then incubated for 45 min at 65°C, extracted with phenol, ethanol precipitated and resuspended in TE. The DNA was end-labelled with polynucleotide kinase (17).

RESULTS

I. Formamide-PERT hybridization

The effect of various PERT conditions on single strand length. Utilizing the PERT hybridization conditions described by Kohne et al. (7), we observed a reduction in the single-strand and double-strand lengths of the DNA following hybridization. We modified

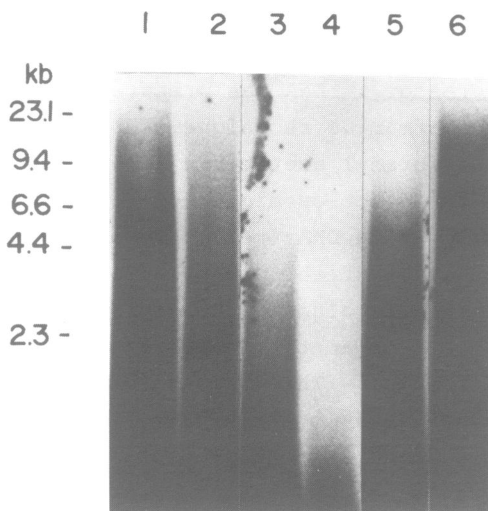

Figure 1. The effect of denaturing and PERT conditions on single DNA strand length. A Bcl I digest of human genomic DNA was labelled by fill-in with Klenow fragment of DNA polymerase I and [α-^{32}P] dATP, denatured with alkali or formamide and hybridized under PERT reaction conditions. An autoradiogram of a 0.8% denaturing agarose gel is shown. Lane 1: genomic DNA denatured in formamide; lane 2: genomic DNA denatured in alkali; lane 3: genomic DNA after PERT (no formamide added) at a DNA concentration of 1 ug/ml; lane 4: genomic DNA after PERT at a DNA concentration of 10 ug/ml lane 5: genomic DNA after PERT reaction in 2% formamide at a DNA concentration of 1 ug/ml; lane 6: genomic DNA after F-PERT reaction (8% formamide) at a DNA concentration of 1 ug/ml. Positions of molecular weight markers (Hind III lambda, in kb) are indicated.

the PERT conditions in an effort to preserve the single-strand length of the 10-20 kb human genomic DNA fragments being hybridized. From other work with plasmid and genomic DNAs (data not shown), we observed that heat denaturation can cause significant single-strand nicking of the DNA. We tried two other methods for denaturing the DNA: formamide and alkali treatment. The DNA used for the experiment was Bcl I-digested human genomic DNA radiochemically labelled with Klenow fragment (17). These denatured DNAs were separated on an alkaline agarose gel to assess the single-strand lengths (Figure 1, lanes 1 & 2). The alkaline-denatured DNA (lane 2) appears to be of a slightly shorter length than the DNA that was denatured with formamide

(lane 1), although both treatments give essentially the same pattern of large single-strand fragments. (The average length of Bcl I-digested human DNA is 2359 bp, see ref. 18).

These denatured DNAs were used for PERT reactions with the following changes in the reaction conditions described by Kohne *et al.* (7). In an effort to minimize shearing the reactions were gently agitated for 20 h at room temperature. The alkaline denatured DNA was reannealed in the presence of 12.5% phenol at DNA concentrations of 1 ug/ml and 10 ug/ml. The DNA denatured in formamide was reannealed in a similar reaction mixture but the presence of formamide from the DNA denaturation gave a final concentration of 2% formamide in the reaction mixture. An additional reaction was done at a DNA concentration of 1 ug/ml in which more formamide was added to the reaction mixture. In this case, the final concentration of formamide was 8%. In order to form an emulsion in the presence of 8% formamide the mixture had to be adjusted to a final concentration of 28% phenol. The DNA molecules which had been reannealed by the PERT procedure were separated on an alkaline agarose gel to determine their single-strand lengths. The autoradiogram of this gel is shown in Figure 1. The DNA that was hybridized under PERT conditions in the absence of formamide was reduced to a shorter molecular length than that treated in the presence of formamide. At the higher DNA concentration (10 ug/ml, lane 4), the single-strand length of the alkaline denatured material was reduced in size to less than 1 kb. At the lower DNA concentration (1 ug/ml, lane 3), the reduction in single-strand length was not as great, although most of the DNA was of a size less than 6 kb. The presence of formamide in the PERT reaction mixture has a major effect on the preservation of DNA length. At a concentration of 2% formamide (lane 5), the single-strand length is larger than that found following the identical treatment without formamide (lane 4) however, the majority of the DNA is still of a single-strand length less than 9 kb. After PERT hybridization in formamide at a concentration of 8% (F-PERT) the single-strand lengths of the DNA (lane 6) were virtually identical to that of the starting DNA (lane 1). Because of the solubility of phenol in formamide, PERT hybridizations with more than 8-10% formamide

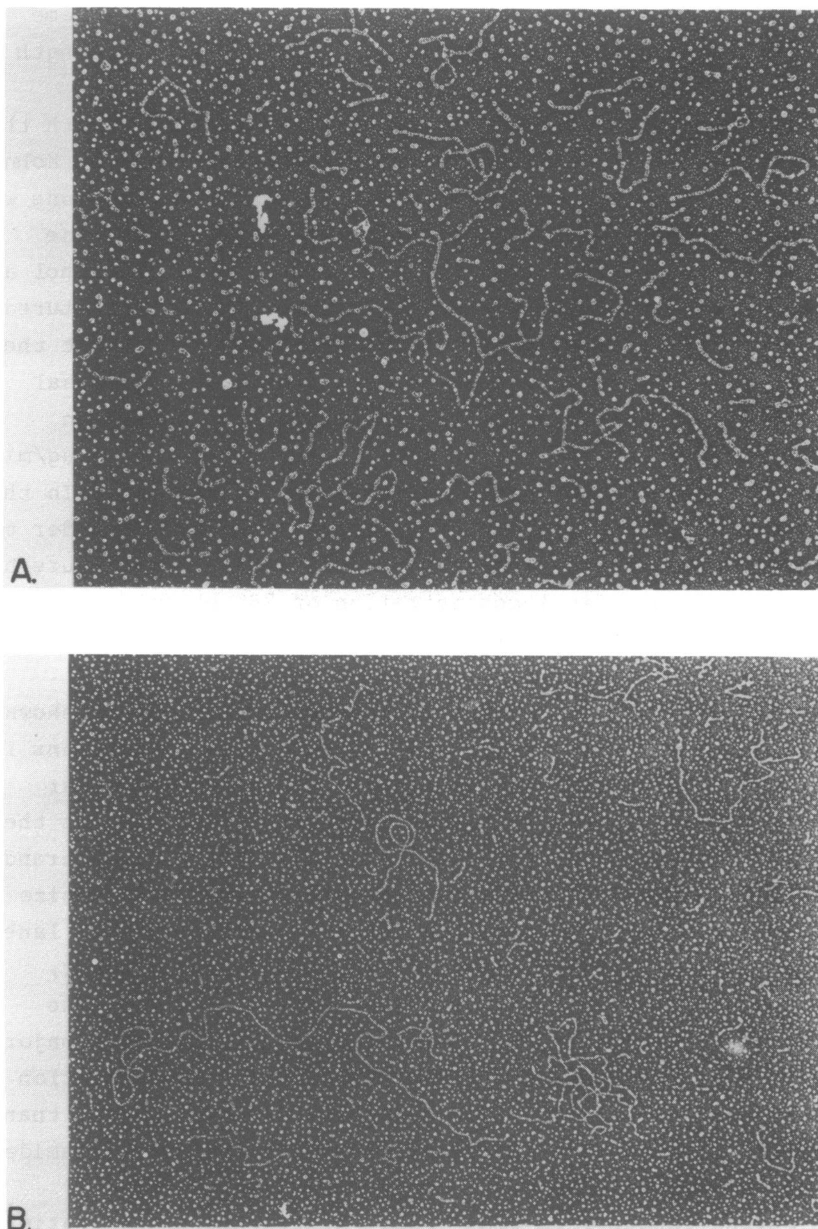

Figure 2. Electron micrographs of of DNA after PERT and F-PERT reactions. Electron micrographs of the reaction products of PERT (A) and F-PERT (B) DNAs at a concentration of 1 ug/ml are shown.

were not achieved. The factor determining the single-strand lengths of the products was not the method of denaturation (alkali or formamide) but rather the presence of formamide in the emulsion mixture (see Discussion). The products of the hybridization reactions (1 ug/ml) after PERT and F-PERT reactions were examined with an electron microscope. Following the standard PERT reaction (Figure 2A) the DNA consisted mostly of short, partially double-stranded molecules. The F-PERT hybridized DNA however, (Figure 2B) consisted mostly of long double-stranded molecules.

Time Course of F-PERT hybridization. The products of the F-PERT reaction were analyzed over time of hybridization to determine their single-strand and double-strand composition. Human genomic DNA was digested with Kpn I (average fragment size 8571 bp, see ref. 18). During the reaction, samples were removed at 0, 0.3 h, 1.5 h, 4.75 h and 22.5 h. One-half of each sample was directly fractionated by agarose gel electrophoresis (Figure 3A). The other half of the sample was digested with S1 nuclease and then fractionated by agarose gel electrophesis (Figure 3B). The ethidium stained pattern of the DNA from the F-PERT reaction without S1 treatment shows the total DNA profile (double and single strands); whereas the S1 resistant DNA is the fraction that is double-stranded. Comparison of the two gels shows that at zero time all the DNA was single-stranded. As F-PERT reaction time increases a larger proportion of the material becomes double-stranded until after 22.5 h the F-PERT reacted DNA had a size profile comparable to that of an undenatured Kpn I digest of human genomic DNA. In addition, over the time course of F-PERT hybridization a considerable amount of S1 sensitive DNA is retained at the origin. This DNA is probably composed of short, hybridized repeats and networks that form rapidly upon hybridization under F-PERT conditions (see Discussion). As the hybridization time increases this DNA becomes incorporated into longer, double-stranded molecules.

Representation of single copy sequences in the F-PERT hybridized population. To determine if a single copy sequence was represented in the reannealed population of DNA molecules, F-PERT hybridized DNA was analyzed by Southern hybridization with a

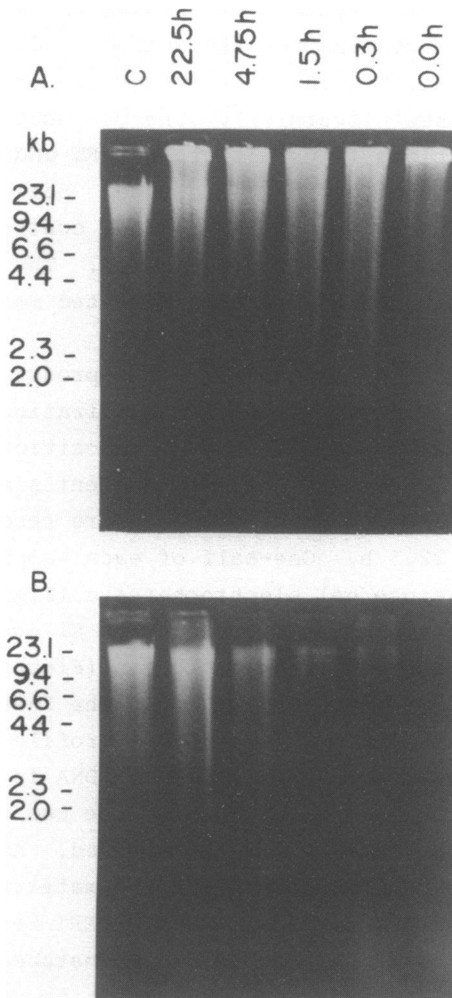

Figure 3. Time course of F-PERT hybridization. A Kpn I digest of human genomic DNA was denatured in alkali and agitated under F-PERT conditions (50 ug/ml), 20 ug samples were removed at the times indicated and run directly on a 0.7% agarose gel (A) or treated with nuclease S1 and run on a 0.7% agarose gel (B). Lane C is a Kpn I digest of human genomic DNA that was never denatured. Positions of molecular weight markers (Hind III lambda, in kb) are indicated.

single-copy sequence probe. Human genomic DNA was cleaved with Eco RI, denatured in alkali and agitated under F-PERT conditions for 21 h at room temperature. The DNA was purified as described

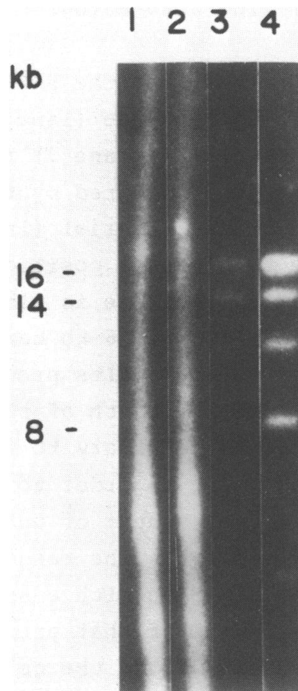

Figure 4. Southern hybridization of F-PERT hybridized human genomic DNA. Human genomic DNA was digested with Eco RI and hybridized under F-PERT conditions (80 ug/ml). A portion of the DNA after F-PERT was treated with nuclease S1 and the samples were separated on a 0.6% agarose gel. The DNA fragments were transferred to a nylon membrane and probed with C_λ (see Results). An autoradiogram of the Southern hybridization is shown. Lane 1: genomic DNA denatured in alkali; lane 2: genomic DNA after F-PERT without S1 treatment; lane 3: F-PERT hybridized DNA treated with S1 nuclease; lane 4: Eco RI digested human genomic DNA. 5 ug of DNA were loaded in each lane. Molecular weights (in kb) are indicated.

in Materials & Methods and a fraction was treated with S1 nuclease to remove any single strands that remained. The DNA was separated on an agarose gel and transferred to a nylon membrane. The membrane was hybridized with a radiochemically labelled probe from the lambda light chain immunoglobulin constant region, C_λ (19). For DNA isolated from germ-line tissues, this probe hybridizes to Eco RI fragments of lengths 16 kb, 14 kb and 8 kb, as well as a small set of variant bands that result from Eco RI

site polymorphisms (20). The autoradiogram of this gel is shown in Figure 4.

The denatured DNA (lane 1) and the F-PERT-hybridized DNA that was not treated with S1 nuclease (lane 2) give similar profiles. Upon treatment with S1 (lane 3) however, bands of hybridization appear that were obscured by the presence of excess single strands in the untreated material (lane 2). The hybridized bands migrate to the same positions as those of the native Eco RI digest (lane 4). There is also no attenuation of the autoradiographic signal of the 16 kb band relative to the 14 and 8 kb bands. Together these results provide evidence that there was no detectable loss in length of the F-PERT-hybridized DNA up to 16 kb. That it was necessary to treat the F-PERT-hybridized DNA with S1 nuclease in order to visualize the bands is evidence that a significant amount of unhybridized DNA remains following a 20 h F-PERT reaction. The range of signal we have detected by Southern hybridization with a single copy probe from F-PERT-hybridized DNA is 20-30% of that predicted from the optical density of the DNA loaded on the gel (not shown).

II. Fractionation of reannealed homoduplex and heteroduplex DNA molecules following differential methylase protection.

To determine whether heteroduplex DNA molecules could be selectively protected from digestion with the two corresponding restriction enzymes by differential methylation we began with a model system. Lambda DNA free of N^6-methyladenine was digested with Hind III and methylated separately with either Dam methylase or Alu methylase. DNA methylated with Dam methylase is resistant digestion by Alu I. Equal amounts of the two methylated DNAs were mixed, denatured and hybridized. The resulting mixture should consist of about one-half heteroduplex molecules and one-half reannealed homoduplex molecules. The DNAs were digested with Alu I and/or Mbo I and separated on an agarose gel (Figure 5A). The ethidium stained pattern showed that the Alu-methylated DNA was resistant to digestion with Alu I (lane 5) but sensitive to digestion with Mbo I (lane 6). Conversely, the DNA methylated with Dam methylase was cleaved by Alu I (lane 1) but resistant to digestion by Mbo I (lane 2). Digestion of a hybridized mixture of Alu and Dam methylated DNAs with both Alu I and Mbo I (lane 3)

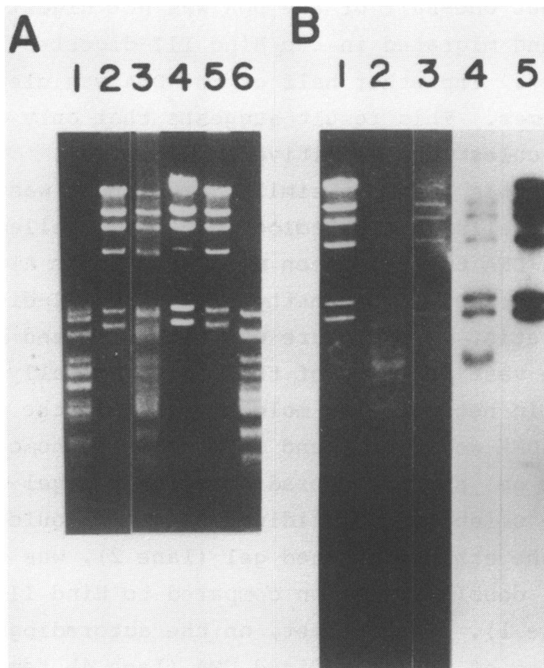

Figure 5. Restriction endonuclease digestion of differentially methylase protected lambda DNA fragments. Lambda DNA free of N^6-methyladenine was digested with Hind III and methylated with either Alu methylase or Dam methylase. (A) Equal amounts of the two methylated species were mixed, denatured and reannealed. The methylated and unmethylated DNAs were cut with Alu I and/or Mbo I and separated on a 1% agarose gel. Dam-methylated Hind III-digested lambda DNA following digestion with Alu I (lane 1) and Mbo I (lane 2) is shown. The hybridized mixture of Alu-methylated and Dam-methylated Hind III-digested lambda DNA is shown following double digestion with Alu I and Mbo I (lane 3). Untreated Hind III-digested lambda DNA is shown in lane 4. Alu-methylated Hind III-digested lambda DNA following digestion with Mbo I (lane 5) and Alu I (lane 6) is shown. (B) Alu-methylated Hind III-digested lambda DNA was end-labelled with polynucleotide kinase and [γ - ^{32}P] ATP and mixed in a 1:10 ratio with Dam-methylated Hind III-digested lambda DNA, denatured and reannealed. The heteroduplex DNA was doubly digested with Alu I and Mbo I and separated on a 1.2% agarose gel. The ethidium stained pattern of this gel is shown in lanes 1-3 and an autoradiogram of the gel is shown in lanes 4-5. Lanes 1 and 5: unmethylated lambda DNA digested with Hind III; lanes 2 and 4: Alu I/Mbo I double digest of the methylated heteroduplex; lane 3: undigested Alu-methylated/Dam-methylated heteroduplex.

showed that about one-half of the DNA was not digested with either enzyme and migrated in the Hind III-digested lambda DNA pattern (lane 4). The other half of the DNA was cleaved by one of the two enzymes. This result suggests that only the homoduplex molecules were sensitive to digestion.

To confirm this result a similar experiment was done with radioactively labelled DNA. Radiochemically labelled Hind III-digested lambda DNA that had been methylated with Alu methylase was mixed with unlabelled Dam-methylated Hind III-digested lambda DNA in a 1:10 ratio. The mixture was denatured and hybridized. In this way the vast majority of the radiochemically labelled DNA would be found in heteroduplex molecules, while the majority of the unlabelled DNA would be found in reannealed homoduplex molecules. The gel and an autoradiogram of the gel are shown in Figure 5B. The unlabelled hybridized DNA that could be visualized on the ethidum stained gel (lane 2), was cleaved after Alu I and Mbo I double digestion compared to Hind III-digested lambda DNA (lane 1). In contrast, on the autoradiogram the labelled fraction of the hybridized DNA (lane 4) remained intact after digestion with Alu I and Mbo I compared to labelled Hind III-digested lambda DNA (lane 5). These results showed that heteroduplex molecules from differentially methylated DNAs were resistant to digestion whereas the reannealed Dam-methylated homoduplex DNA molecules were cleaved by Alu I.

A similar experiment was performed with human genomic DNA. Eco RI-digested human genomic DNA was methylated separately with either Alu methylase or Dam methylase. A mixture of equal amounts of the two methylated DNAs was denatured with alkali and agitated under F-PERT conditions for 22 h at room temperature. The DNA was doubly digested with Alu I and Mbo I and treated with S1 nuclease (Figure 6). An F-PERT hybridization of unmethylated Eco RI-digested DNA is shown in lane 1. Most of the DNA was larger than 2 kb. When this DNA was cleaved with Alu I and Mbo I (lane 2) the fragments had an average size well below 1 kb. The F-PERT reaction of the Alu- and Dam-methylated DNAs is shown in lane 3. When this DNA was cleaved with Alu I and Mbo I (lane 4) some of the fragments also migrated below 1 kb but a similar amount remained in the high molecular weight region. This result

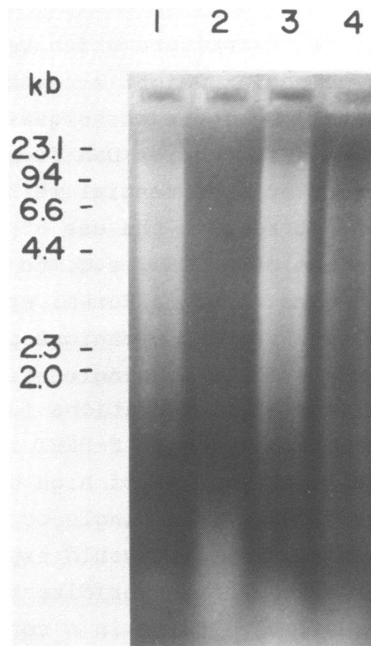

Figure 6. Restriction endonuclease digestion of differentially methylase protected and F-PERT hybridized human DNA. Human genomic DNA was digested with Eco RI and methylated with either Alu methylase or Dam methylase. Equal amounts of the methylated DNAs were mixed, denatured and hybridized under F-PERT conditions. The reaction products from methylated and unmethylated DNAs were digested with Alu I and Mbo I, treated with S1 nuclease and separated on a 0.6% agarose gel. The unmethylated Eco RI-digested DNA after F-PERT (lane 1) and digested with Alu I and Mbo I (lane 2); and methylated DNAs after F-PERT (lane 3) and digested with Alu I and Mbo I (lane 4) are shown. Molecular weight markers (Hind III lambda, in kb) are indicated.

suggests that as in the model system following differntial methylase protection, reannealed genomic homoduplex molecules are cleaved with Alu I or Mbo I whereas genomic heteroduplex molecules are resistant to cleavage. Since about one-half of the hybridized DNA molecules are expected to be of the heteroduplex type, this result also showed that the denaturation conditions prior to F-PERT hybridization were sufficient to allow random reassortment of single strands into heteroduplex and homoduplex molecules, even for high molecular weight fragments.

DISCUSSION

We have presented two techniques which we have linked in order to isolate high molecular weight heteroduplex DNA. Depending on experimental goals the techniques are separable. For example, analysis of heteroduplex DNA from cloned sequences could be made much easier by differential methylase protection and such analysis does not require the use of F-PERT. Counter examples exist for the use of F-PERT, e.g. to isolate DNA clones that span a deletion as described by Kunkel et al. (8).

The modifications of the PERT technique presented here substantially reduce the problem of single strand cleavage that occurred using the original PERT conditions (7). We have found that the presence of formamide in the F-PERT reaction mixture results in an increase in the number of high molecular weight hybridized DNA molecules containing single copy sequences. We do not understand why this occurs. One would expect that during the PERT reaction repetitive sequences hybridize first. Many of the repeated DNA sequences will hybridize in a context where the adjacent sequences are non-homologous. The results presented in Figure 3 are consistent with the formation of such networks. In order to generate long double-stranded molecules containing correctly hybridized single-copy regions, the hybridized repeats must be displaced. Formamide might be thought to play a role in helping to denature the short repetitive DNA duplexes. Evidence against this explanation is the inability to replicate the formamide effect upon the substitution of 1-25% of the denaturant DMSO for formamide in the PERT hybridization mixture (data not presented). The ineffectiveness of adding DMSO (or up to 10% sucrose) in improving PERT hybridization (data not shown) also argues against a major role for viscosity in preserving single-strand length. Perhaps significant is the appearance of the emulsion upon the addition of formamide, which is noticeably different from an emulsion containing only phenol. The emulsion with formamide is milky-white which reflects a much finer emulsion than that which forms in the presence of phenol alone. We suggest that this change, which does not occur upon addition of sucrose or DMSO, alters the nature of the organic/aqueous interface and its surface area. This interface is presumably the

site of hybridization with the PERT technique (7, 21). Perhaps the addition of formamide increases the surface area and/or somehow reduces the shearing forces on the single-stranded DNA. Additional evidence suggests it is single-stranded DNA which is vulnerable to cleavage during PERT. First the length of undenatured double-stranded DNA agitated under PERT hybridization conditions is maintained even in the absence of formamide (data not shown). Secondly, we routinely obtain only 20-30% S1 resistant hybrid following F-PERT hybridization with a size profile similar to that of the input DNA. Times of hybridization longer than 24 h do not increase this yield. We believe that incomplete hybridization reflects single-strand cleavage during the F-PERT hybridization as long F-PERT hybridization times (40-80 h) generate similar amounts of S1 resistant DNA in the high molecular weight region while showing an accumulation of S1 resistant DNA fragments of a size less than 2 kb (data not shown).

The basis for fractionation of heteroduplex DNA molecules following differential methylase protection is straight forward by comparison. The number of methylases available for this application is fairly large (>10) and may increase in the future. Occasionally, we have seen incomplete protection of both strands in long heteroduplex molecules. Therefore, it is important to test the methylated double-stranded DNA for resistance to digestion with the appropriate restriction enzyme before denaturing and reannealing. Double treatment with SAM as described in Materials and Methods should overcome this problem with most if not all methylases. Separation of the cleaved homoduplex DNA molecules from the large undigested heteroduplex DNA molecules can be achieved in several ways. In the example presented here we would expect the sizes of the cleaved homoduplex molecules to be fairly small following double digestion with Alu I and Mbo I. For human DNA, over 99% of the DNA fragments generated by digestion with Alu I and over 95% of those generated by Mbo I are of a size less than 1 kb (18). Physical separation based on size has been achieved on gels. Gradient fractionation is also possible (17). Biological separation can be employed by cloning the fragments into lambda

vectors that require a 10 - 22 kb insert for viability. Following ligation of the heteroduplex/homoduplex mixture into the appropriate lambda vector few if any of the recombinants containing homoduplex fragments will be viable. Therefore the vast majority of the resulting genomic library will be composed of fragments which are derived from the heteroduplex population. The fractionated heteroduplex DNA molecules can be further purified by reaction with mismatch specific agents (3). Cloning experiments using F-PERT hybridized, Mbo I and Alu I-resistant methylated human DNA, have been successful following size fractionation (data not shown). The yield of DNA after all of these steps is about 0.5% of the starting DNA, yet we have produced phage libraries with yields in terms of plaques/ng DNA which are similar to those obtained with restriction enzyme digested human DNA. We are currently characterizing these recombinant libraries.

Instead of cloning, the "tagged" heteroduplex molecules could be reacted with immunologic reagents directed against the mismatch-specific agent and subjected to affinity chromatographic purification. Alternatively, if Dam methylase is replaced by another methylase, large DNA inserts containing some mismatches may be removed by mismatch-stimulated killing (22).

The methods described here used in combination with "tagging" by mismatch-specific agents (3) should allow the use of heteroduplex human DNA as a reagent for obtaining DNA markers linked to genetic diseases and for creating subregional chromosome maps. The rationale for such an approach is discussed in the preceeding paper (1).

Acknowledgements We thank Barbara Cacciapaglia and John Neuweiler for excellent technical assistance, Nancy Rubicco, Theresa Guarino and Karen Kaminsky for help in preparation of the manuscript and Drs. Nadrian Seeman and Neville Kallenbach for reviewing the manuscript.

REFERENCES

1. Sanda, A.I. and Ford, J.P. (1986) Nucleic Acids Res. 14, 7265-7283.
2. Dulbecco, R. (1986) Science, 231, 1055-1056.
3. Novack, D.F., Casna, N.J., Fischer, S.G. and Ford, J.P. (1986) Proc. Natl. Acad. Sci. USA 83, 586-590.

4. Myers, R.M., Larin, Z. and Maniatis, T. (1985) Science, 230, 1242-1246.
5. Fischer, S.G. and Lerman, L.S. (1983) Proc. Natl. Acad. Sci. USA 80, 1579-1583.
6. Wallace, R.B., Schold, M., Johnson, M.J., Dembeck, P. and Itakura, K. (1981) Nucleic Acids Res. 9, 3647-3656.
7. Kohne, D.E., Levison, S.A. and Byers, M.J. (1977) Biochemistry, 16, 5329-5341.
8. Kunkel, L.M., Monaco, A.P., Middlesworth, W., Ochs, H.D. and Latt, S.A. (1985) Proc. Natl. Acad. Sci. USA 82, 4778-4782.
9. Gruenbaum, Y., Cedar, H. and Razin, A. (1981) Nucleic Acids Res. 9, 2509-2515.
10. Gelinas, R.E., Myers, A.P. and Roberts, R.J. (1977) J. Mol. Biol. 114, 169-179.
11. White, R., Leppert M., Bishop, D.T., Baker, D., Berkowitz, J., Brown, C., Callahan, P., Holm, T. and Jerominski, L. (1985) Nature, 313, 101-105.
12. McDonnell, M.W., Simon, M.N. and Studier, F.W. (1977) J. Mol. Biol., 110, 119-146.
13. Mears, J.G., Ramirez, F., Leibowitz, D., Nakamura, F., Bloom, A., Konotey-Ahulu, F. and Bank, A. (1978) Proc. Natl. Acad. Sci. USA 75, 1222-1226.
14. Southern, E.M. (1975) J. Mol. Biol. 98, 503-507.
15. Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237-251.
16. Davis, R.W., Simon, M. and Davidson, N. (1971) in Methods in Enzymology, Grossman, L. and Moldave, L., Eds., Vol. 21, pp. 413-428, Academic Press, New York.
17. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
18. Bishop, D.T., Williamson, J.A. and Skolnick, M.H. (1983) Am. J. Hum. Genet. 35, 795-815.
19. Arnold A., Cossman, J., Bakhshi, A., Jaffe, E.S., Waldmann, T.A. and Korsmeyer, S.J. (1983) N. Engl. J. Med. 309, 1595-1599.
20. Heiter, P.A., Hollis, G.F., Korsmeyer, S.J., Waldmann, T.A. and Leder, P. (1981) Nature, 294, 536-540.
21. Wieder,R. and Wetmur, J.G. (1982) Biopolymers, 21, 665-677.
22. Doutriaux, M.P., Wagner, R. and Radman, M. (1986) Proc. Natl. Acad. Sci. USA 83, 2576-2578.