Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 2720–2724, April 1990
Genetics

A method for difference cloning: Gene amplification following subtractive hybridization

(polymerase chain reaction/inherited disease/cancer/infectious disease)

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Contributed by Michael Wigler, January 2, 1990

ABSTRACT **We describe a procedure for genomic difference cloning, a method for isolating sequences present in one genomic DNA population ("tester") that is absent in another ("driver"). By subtractive hybridization, a large excess of driver is used to remove sequences common to a biotinylated tester, enriching the "target" sequences that are unique to the tester. After repeated subtractive hybridization cycles, tester is separated from driver by avidin/biotin affinity chromatography, and single-stranded target is amplified by the polymerase chain reaction, rendering it double-stranded and clonable. We model two situations: the gain of sequences that result from infection with a pathogen and the loss of sequences that result from a large hemizygous deletion. We obtain 100- to 700-fold enrichment of target sequences.**

One common and fundamental problem of molecular biology confronts us when two similar genomes differ and we desire to understand the difference. One simple form of this problem can occur when a genome becomes deleted for sequences present in another due to germ-line mutation, as can happen in genetic disease (1–3), or due to somatic mutation, as can happen during the development of cancer (4–6). Differences can also be acquired by infection with a DNA-based pathogen. Methods for identifying and isolating sequences present in one DNA population that are absent or reduced in another are called "difference cloning." Methods for difference cloning in cDNA populations have been widely described (7–10). Only one method for the difference cloning of genomic DNA is reported in the literature. This method was first described by Lamar and Palmer (11), who used it to clone sequences from the Y chromosome. Kunkel *et al.* (12) used a variation of this method to clone fragments of the Duchenne muscular dystrophy (DMD) locus, which becomes deleted in some afflicted individuals. Clones from other X-chromosome linked deletions have also been obtained by this method (13). We describe here another method for genomic difference cloning that is at least as powerful. Although our method is not yet sufficiently powerful to isolate and define the small differences in genomes that would make it enormously useful as a tool for the study of neoplasia or infectious disease of viral origin, improvements are possible that could bring our method into that range. In its present state our method is useful for the analysis of some genetic diseases and infectious diseases of unknown origin.

We make frequent use of the following nomenclature. Two DNA populations that differ are referred to as "tester" and "driver." Tester contains "target" sequences that are not present in driver. In the procedure of Lamar and Palmer (11), target sequences were enriched relative to the remainder of tester in the following way. Tester DNA was prepared by cleavage with *Sau*3A and mixed with an excess amount of randomly sheared driver DNA. DNAs were melted and reannealed to high C_0t values. Double-stranded DNA (dsDNA) was then cloned into the *Bam*HI site of a cloning vector. In principle, only tester DNA annealed to itself would be clonable into a *Bam*HI site. Neither driver annealed to itself, nor tester to driver, would be clonable. Thus cloned material would be enriched in target since it can form duplex only with itself. The yield of this method is poor, since it depends upon the slow reannealing of dilute tester to itself, and the theoretical enrichment cannot exceed the mass ratio of driver to tester. Yields can be improved through the use of accelerated annealing conditions, such as the phenol emulsion reannealing technique (12–14).

Our procedure utilizes a different form of subtractive hybridization (see Fig. 1). Tester DNA is specially prepared (cleaved, biotinylated, and ligated to "template" oligonucleotides). Prepared tester is then mixed with an excess of randomly sheared driver, melted, and annealed. After annealing proceeds to 90% completion for driver, the remaining single-stranded DNAs (ssDNAs) are isolated. ssDNA will contain tester that is relatively enriched for target sequences, since target sequences will not have had time to reanneal. Unreannealed driver will also be present in great excess. This procedure is reiterated twice more by the addition of fresh driver, melting, annealing, and fractionation. Tester highly enriched in target sequences is then completely separated from the excess unhybridized driver by the presence of the biotin group. The resulting tester is amplified by a polymerase chain reaction (PCR) (15) and cloned. Using this procedure we obtain 100- to 700-fold enrichment of target sequences. In the following we describe the procedure in detail and demonstrate its use for two model systems.

MATERIALS AND METHODS

Preparation of Driver and Tester DNA. Purified high molecular weight DNA serving as driver was sheared by sonication to a size range of 0.5–2.0 kilobase pairs (kbp). The sheared DNA was size fractionated on a Sepharose 4B (Pharmacia) column to exclude low molecular weight material. Aliquots containing 410 μg of the size-selected driver were ethanol precipitated and stored at −20°C until used. Tester, derived from high molecular weight placental DNA mixed with bacteriophage λ DNA (New England Biolabs), was cleaved with *Sau*3A (New England Biolabs) using the conditions recommended by the supplier and size fractionated on a Sepharose 4B column. Fractions containing fragments between 300 base pairs (bp) and 2 kbp were pooled and concentrated by ethanol precipitation. DNA was resuspended in TE (1 mM Tris·HCl, pH 7.5/1 mM EDTA), 20 μg of the cleaved tester was added to 30 μl of buffer containing

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Abbreviations: DMD, Duchenne muscular dystrophy; PCR, polymerase chain reaction; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; HAP, hydroxylapatite.

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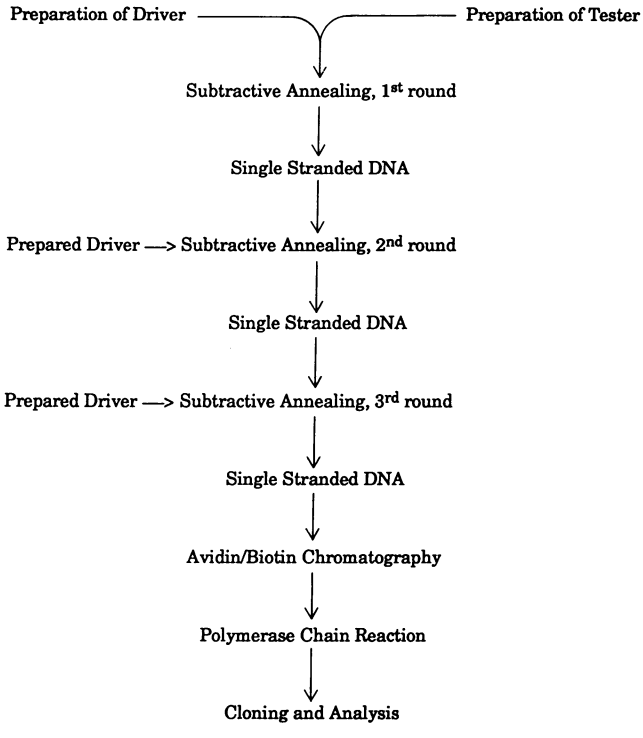

FIG. 1. Flow diagram for genomic difference cloning.

50 mM NaCl, 10 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 500 μM (each) dATP, dGTP, and dCTP, and 20 μM Bio-12-SS-dUTP (16) (a gift from R. Pergolizzi, North Shore Hospital), and the Klenow fragment of *Escherichia coli* polymerase I (5 units, New England Biolabs) was added to fill in the 5′ protruding ends. The blunt-ended and biotinylated DNA fragments were then ligated to the oligonucleotide template: 5′-CTTACCATGGTAAG-3′. The oligonucleotides were synthesized on an Applied Biosystems 380-A DNA synthesizer and were purified by polyacrylamide gel electrophoresis. Oligonucleotides were self-annealed and phosphorylated; kinase and ligation reactions (17) were performed in buffers recommended by suppliers except that dithiothreitol was omitted to avoid cleavage of the biotin group. Oligonucleotides that ligated together were subsequently monomerized by cleavage at the *Hin*dIII site that forms upon self-ligation. Again, dithiothreitol was omitted in the restriction enzyme buffer. The free oligonucleotide templates were removed by refractionation on a Sepharose 4B column. The fractions containing the tester DNA were pooled, ethanol precipitated, and resuspended in 10 μl of TE. The DNA concentration was determined by absorption at 260 nm and by ethidium bromide staining in agarose gels. The tester DNA was stored in TE at −70°C. Tester was stable for about 4 weeks.

Solution Hybridization. Tester and driver DNA were mixed at a mass ratio of 1:200 to a final concentration of 8.2 mg/ml of driver. The hybridization was performed in a volume of 50 μl in buffer containing 120 mM sodium phosphate (pH 6.8) overlaid with mineral oil (Sigma) to avoid evaporation. The DNA mixture was heat-denatured at 97°C for 10 min followed by annealing in the presence of 0.8 M NaCl at 64°C for 36 hr. After this time ≈90% of the driver DNA was reannealed to form dsDNA as determined by fractionation over hydroxylapatite (HAP). The annealing mix was diluted 1:300 in 120 mM sodium phosphate (pH 6.8) and incubated with HAP (1 mg/μg of DNA; DNA-grade Bio-Gel HTP, Bio-Rad) in a batch procedure (18) with the following modifications. Incubation of the mix was at 64°C for 20 min. ssDNA was recovered by centrifugation for 15 s at 1500 × *g*. The supernatant was collected and recentrifuged at 10,000 × *g* for 30 s. The volume of the HAP supernatant was reduced by precipitation of the DNA with glassmilk (Geneclean kit, Bio 101, La Jolla, CA) according to the manufacturer's recommendations. The ssDNA was eluted from the glassmilk in a volume of 40 μl of H_2O at 65°C for 10 min, and subsequently sodium phosphate (pH 6.8) was added to a final concentration of 120 mM. This eluate was used to redissolve an aliquot (410 μg) of the ethanol-precipitated driver. The DNAs were overlaid with mineral oil and the next round of solution hybridization was started.

Avidin/Biotin Affinity Chromatography. Following the last solution hybridization, avidin/biotin affinity chromatography was performed (16). The eluate of the glassmilk was adjusted to 200 mM NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA in a volume of 100 μl, and avidin DN (Vector Laboratories) was added in excess (1 μg) to the recovered ssDNA population. After incubation for 30 min at room temperature, the mixture was chromatographed on a 100-μl biotin-cellulose (Pierce) column. The column flow-through was cycled three times followed by washes with the loading buffer (see above), loading buffer at pH 8.5, and a buffer containing 50 mM NaCl, 10 mM Tris·HCl (pH 8.5), and 1 mM EDTA. The latter buffer including 50 mM dithiothreitol and 5 ng of high molecular weight carrier DNA per ml was used to elute the bound DNA in 100-μl fractions.

PCR and Cloning. One-fourth of each of the eluates from the biotin-cellulose column was subjected to amplification by PCR (15). The PCR contained, in a volume of 50 μl, 50 mM KCl, 10 mM Tris·HCl (pH 8.4), 3 mM $MgCl_2$, 0.01% gelatin, 200 μM (each) dATP, dCTP, dGTP, and dTTP, 100 pmol of the PCR oligonucleotide primers (synthesized on an Applied Biosystems 380-A DNA synthesizer), and 1 unit of *Thermus aquaticus* (Taq) DNA polymerase (Perkin–Elmer/Cetus). The PCR primer was 5′-CTTACCATGGTAAGGATC-3′. The cycling conditions were 1.5 min of denaturation at 95°C, 3 min of annealing at 55°C, and 7 min of polymerization at 72°C. The cycle was repeated 50 times (Thermocycler, Perkin–Elmer/Cetus). The last cycle had an additional extension at 72°C for 10 min. After phenol extraction and ethanol precipitation, the amplified material was digested with *Sau*3A, fractionated on a Sepharose 4B column to remove low molecular weight products, concentrated by ethanol precipitation, and ligated to *Bam*HI-cleaved and alkaline phosphatase-treated pUC118 vector (19). Competent *E. coli* DH5α (20) were transformed with the ligation mix and selected for ampicillin resistance. Recombinant clones were picked and analyzed based on the blue/white color distinction.

General Methods. High molecular weight DNA from human placenta and from cell lines was isolated as described (21, 22). Plasmid DNA was isolated by the alkaline lysis procedure (23). For Southern blotting (24), restriction endonuclease-digested genomic DNA (10 μg), digested plasmid DNA, and PCR products (one-fifth of the reaction) were fractionated on agarose gels. The DNAs were transferred onto GeneScreen*Plus* nylon membrane (NEN), using a vacuum blotting unit (Vacugene, LKB) for 0.8% gels. λ DNA to be used as a hybridization probe was labeled *in vitro* using a nick-translation kit (BRL). ^{32}P-labeled single-strand DNA probes were prepared from M13 single-strand templates as described (25). Hybridizations to labeled probes were performed in a volume of 30 ml at 68°C overnight, and hybridization and washing buffers were as described (26). Autoradiography was performed at −70°C using Kodak XAR-5 films and intensifying screens (27). For rehybridization experiments, probes were stripped from the nylon membrane by incubation in 0.2 M NaOH at 42°C for 30 min.

Cell Lines and Human Placenta. The Epstein–Barr virus-immortalized lymphoid cell line DRL 484 (a gift from T.

Caskey, Baylor College) was maintained in RPMI medium supplemented with 10% fetal calf serum (GIBCO). The human–chinese hamster somatic cell hybrids GM06318B, with a human X chromosome, and GM07297, with human chromosomes 3 and X, were from the Human Genetic Mutant Cell Repository (Camden, NJ). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum. Two passages before extraction of the DNA the cells were subjected to selection in hypoxanthine/aminopterin/thymidine medium. Chinese hamster ovary cells (a gift from D. Spector, Cold Spring Harbor Laboratory) were cultured in F12 medium supplemented with 10% fetal calf serum. Human placenta of a female newborn (a gift from S. Chao, North Shore Hospital) was immediately dissected into small pieces, quickly frozen in liquid nitrogen, and stored at −70°C.

PROTOCOL DESIGN

Preparation of Driver and Tester. High molecular weight driver DNA is sheared by sonication to an average size of 800 bp. High molecular weight tester DNA is cleaved with *Sau*3A and the 5′ overhang is filled in with the Klenow fragment of the *E. coli* DNA polymerase I in the presence of dATP, dGTP, dCTP, and Bio-12-SS-dUTP, a biotinylated analog of dTTP (16). The biotin moiety is linked to the uracil residue by way of a hydrolyzable disulfide group. The tester is thus flush ended and labeled with biotin. Next, double-stranded oligonucleotide template treated with polynucleotide kinase is ligated to tester. The sequence of the oligonucleotide (see *Materials and Methods*) is self-complementary. When the template oligomers ligate to themselves, they form a *Hin*dIII cleavage site. After the ligation of the oligomers to tester, the reaction mix is cleaved with *Hin*dIII to destroy the products of oligomer self-ligation. This step is necessary in order that the subsequent PCR (see below) work efficiently.

Subtractive Hybridizations. Tester and driver are mixed at a mass ratio of 1:200. The DNA mixture is heat-denatured and allowed to reanneal until ≈90% of driver will have formed duplex DNA. DNA is then fractionated by HAP. The single-strand fraction will contain tester sequences enriched in target and a vast excess of unreannealed driver DNA. After the addition of fresh driver DNA, this step is repeated two more times. Each round of annealing and fractionation can, in theory, enrich target over the remainder of tester by the factor $1/(1 - F_i)$, where F_i is the fraction of driver that reanneals in the ith cycle (and assuming tester anneals to driver with the same kinetics as driver). Thus, if driver achieves 90% reannealing in each of three rounds, target will be enriched by 1000-fold.

Separation of Tester from Driver. DNA from the subtractive hybridization steps is heavily contaminated with unreannealed driver. To isolate tester from driver, the single-strand DNA fraction from the third round of annealing is subjected to biotin/avidin affinity chromatography. This step reduces the amount of driver by a factor of 10^4 to 10^5.

Amplification and Cloning of Target. After the affinity purification of tester, it is single stranded and present in very low concentration, due to losses of material occurring at each step of the procedure. To amplify the product of the procedure and to render it double stranded, we utilize the PCR. A PCR primer (see *Materials and Methods*) is added, and DNA is synthesized for 50 cycles. The PCR primer is complementary to the template oligonucleotide and in addition contains the *Sau*3A cleavage sequence at its 3′ end. The product of the PCR reaction is cleaved by *Sau*3A and ligated into the *E. coli* plasmid vector pUC118. *E. coli* strain DH5α is transformed and selected on ampicillin plates containing isopropyl β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. Recombinant clones are identified by the blue/white color distinction.

RESULTS

To test our protocol we used two model systems. In the first, we modeled the gain of information in which a human cell is infected by a pathogen present at about one copy per cell. Tester DNA and driver DNA came from the identical human placenta but bacteriophage λ DNA was added to tester as a known target, in a mass ratio of 1 part per 10^5. Thus the molar ratio of λ sequences to unique human sequences in tester was about 1:1. In the second model system, we chose DNA from a male patient with DMD (28) as driver. This patient has a deletion of about one megabase of DNA from the X chromosome. Tester DNA was derived from the placenta of a female newborn. This system is representative of the case of loss of information.

Gain of Information. λ DNA was added as target at single copy levels to placental DNA. The same placental DNA without λ was used as driver. Several independent trials were performed. The PCR product was electrophoresed in agarose gels and Southern blotted using nick-translated λ DNA as probe. Independent PCR products, even from the same subtraction, contained a random representation of *Sau*3A-cleaved λ fragments (Fig. 2). We do not understand this phenomenon. We also observe a strong preference for the amplification of smaller λ *Sau*3A fragments. PCR products were ligated into vector pUC118 and used to transform DH5α. Approximately 10^4 recombinant clones were obtained. Several hundred white colonies (i.e., those with vectors containing inserts) were picked and pooled in groups of 10. Plasmid DNAs were prepared from these pools, cleaved to release the insert from the vector, and analyzed by Southern blotting for the presence of λ sequences. From our results we calculate that we achieved a 300- to 750-fold enrichment of λ sequences in three independent trials (see Table 1).

Loss of Information. In the second system, tester came from placental DNA and driver came from Epstein–Barr virus-immortalized lymphocytes of a patient with DMD. We

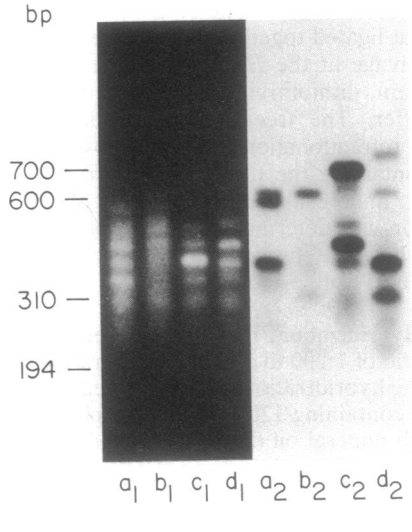

FIG. 2. Independent PCRs from a subtractive hybridization. Human placental DNA was used as driver and human placental DNA supplemented with one copy of bacteriophage λ per haploid genome was used as tester (experiment 1 of Table 1). After subtractive hybridization four independent PCRs were performed. Lanes a_1–d_1, ethidium bromide staining of PCR products electrophoresed on 1.5% agarose gels. Lanes a_2–d_2, Southern blot of a_1–d_1 using nick-translated λ DNA as a probe. Sizes (bp) are indicated on the left.

Table 1. Estimates of enrichment for target in independent subtraction hybridization experiments

Experiment	Target	Complexity of target, kbp	Copies of target per haploid tester	Yield*	Enrichment†
1	λ	50	1.0	5/400	750
2	λ	50	2.0	1/100	300
3	λ	50	2.0	1/100	300
3	DMD locus	1000	1.0	2/50	120

*Number of clones with inserts from target/number of recombinant clones examined.
†Enrichment (E) is calculated by formula $E = (YC_e/RC_a)$, where Y is the yield of target, C_e is the complexity of haploid tester, R is the number of copies of target per haploid tester, and C_a is the complexity of target. We assume the haploid human genome to be 3×10^6 kbp.

also added λ DNA to tester, at two copies per genome, in order to independently monitor the degree of enrichment. The PCR product was again cloned into pUC118 and analyzed. One hundred recombinant clones were picked. Eighty-seven contained detectable inserts, ranging in size from 100 to 700 bp. One clone contained λ sequences. Fifty clones were picked and used individually as probes in Southern blots of cleaved DNA from driver and tester (see Fig. 3). Forty clones appeared to detect single-copy human DNA. Four clones detected a more complex pattern of bands, four detected a diffuse smear or highly repetitive sequences, and two detected no sequences. Two of the 40 clones, with inserts of 350 and 500 bp, hybridized to tester but failed to hybridize to DNA from the patient with DMD. These two clones were then hybridized to a panel of DNAs that included DNA derived from human–hamster hybrid cells retaining only the human chromosome X (Fig. 4). These studies indicate that the human DNA in these two clones contains sequences derived from the X chromosome. We therefore conclude that this DNA was deleted from the DMD locus. We estimate from these results that the target sequences in tester were enriched at least 100-fold (see Table 1).

DISCUSSION

We have described a general procedure for genomic difference cloning and modeled it for two situations: the first in which one DNA population contains acquired sequences, and the second in which one DNA population has lost sequences. In a mathematical sense, both situations are equivalent. Our protocol employs iteration of cycles of subtractive hybridization during which a large excess of driver DNA is used to remove sequences common to the tester DNA, thereby enriching target sequences unique to tester. The specific experimental features that make this approach possible are (*i*) the use of the PCR procedure to amplify low yields of target DNA and convert it to a double-stranded, clonable form and (*ii*) the use of biotinylation of the tester to facilitate its subsequent separation from driver after subtractive hybridization.

With this procedure we achieve about 100- to 700-fold enrichment of target sequences, which is close to the theoretical prediction. This degree of enrichment makes our procedure useful for the analysis of mammalian genomic DNA when the complexity of the target is on the order of a few megabases. Such a situation arises when we desire to isolate sequences lost in large hemizygous or homozygous deletions or when we desire to obtain probes from tissues infected with nonviral pathogens. Our procedure should also be applicable to the problem of small deletions occurring in organisms of low genomic complexity. Our procedure is not yet sufficiently powerful to be readily applied to other problems, such as the cloning of sequences lost from small homozygous deletions found in some tumor cells, or the isolation of probes from tissues infected with unknown viruses.

Several improvements in the basic methodology may be envisioned. In particular, given an enrichment of target, it should be possible to exploit the second-order kinetics of self-annealing to engineer a second stage of purification that

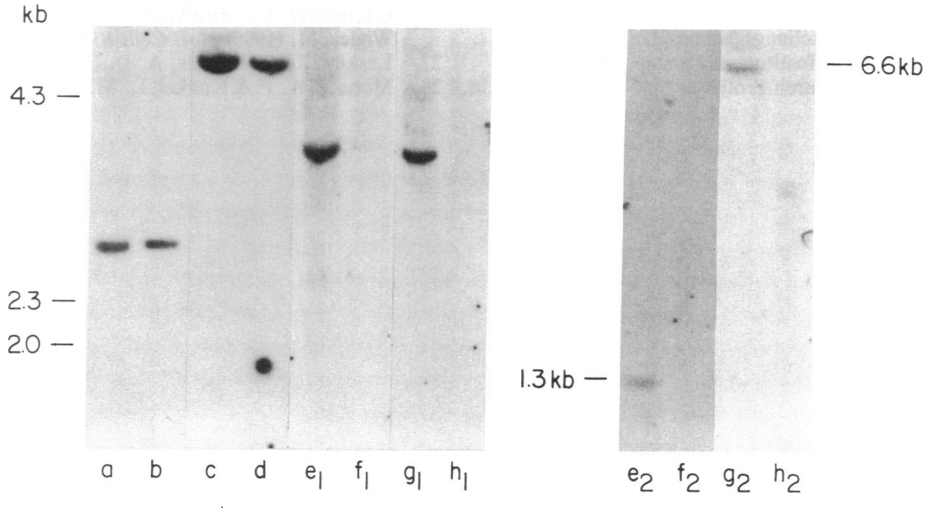

FIG. 3. Southern blots with candidate target sequences as probes. Southern blots of human placental tester DNA (lanes a, c, e, and g) and of the DMD cell line DRL484 driver DNA (lanes b, d, f, and h) were performed with candidate target clones as probes. Genomic DNA (10 μg) cleaved with *Eco*RI (lanes a–d and e_1–h_1) and *Hin*dIII (lanes e_2–h_2) was hybridized with ^{32}P-labeled single-strand probes obtained from clones of the DMD subtraction hybridization library (experiment 3 of Table 1). Lanes a and b and lanes c and d were probed with representative clones recognizing single-copy sequences in both tester and driver. Lanes e and f were probed with difference clone p484-67, which hybridizes to tester DNA (lanes e) only. Lanes g and h were probed with difference clone p484-98, which hybridizes to tester DNA (lanes g) only. The DNA was separated on 0.8% agarose gels. Sizes (kb) are indicated.

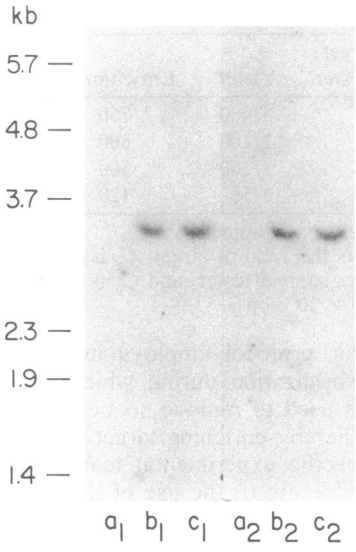

FIG. 4. Southern blots of human–rodent cell hybrids. ^{32}P-labeled single-strand probes of the DMD difference clones p484-67 (lanes a_1–c_1) and p484-98 (lanes a_2–c_2) were hybridized to *Eco*RI-cleaved DNA (10 μg) from the cell lines GM06318B containing only the human chromosome X (lanes b_1 and b_2) and GM07297 containing the human chromosomes X and 3 (lanes c_1 and c_2). DNA from Chinese hamster ovary (lanes a_1 and a_2) served as a control. The DNA was separated on 0.8% agarose gels. Sizes (kb) are indicated on the left.

would further enrich target. The theoretical enrichment for a procedure based on the velocity of self-annealing is an additional N fold, where N is the fold enrichment of target achieved after the subtractive hybridization cycles. The present obstacle to applying this approach is that the product from the PCR process appears to be a stochastic sampling of DNA molecules rather than a uniform amplification of molecules present in tester prior to PCR (see Fig. 2).

We thank Drs. T. Caskey and D. Spector for providing cell lines, Dr. S. Chao for providing placentas, Dr. R. Pergolizzi for providing biotinylated dUTP, and Dr. R. Axel for useful suggestions and a critical reading of the manuscript. Also we thank P. Renna and J. Duffy for the photography and art work and P. Bird for preparing this manuscript. This work was supported by grants from the National Institutes of Health and the American Cancer Society. I.W. is supported by the Deutsche Forschungsgemeinschaft, G.B. was supported by the National Cancer Institute of Canada, and M.W. is an American Cancer Society Research Professor.

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