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Genetics

# Genomic subtraction for cloning DNA corresponding to deletion mutations

**(biotin:avidin affinity purification/deficiency/polymerase chain reaction)**

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ABSTRACT **We have developed a technique, called genomic subtraction, for isolating the DNA that is absent in deletion mutants. The method removes from wild-type DNA the sequences that are present in both the wild-type and the deletion mutant genomes. The DNA that corresponds to the deleted region remains. Enrichment for the deleted sequences is achieved by allowing a mixture of denatured wild-type and biotinylated mutant DNA to reassociate. After reassociation, the biotinylated sequences are removed by binding to avidin-coated beads. This subtraction process is then repeated several times. In each cycle we hybridize the unbound wild-type DNA from the previous round with fresh biotinylated deletion mutant DNA. The unbound DNA from the final cycle is ligated to adaptors and amplified by using one strand of the adaptor as a primer in the polymerase chain reaction. The amplified sequences can then be used to probe a genomic library. We applied genomic subtraction to a yeast strain that has a 5-kilobase deletion, corresponding to 1/4000th of the genome. In the experiment reported here, three rounds of subtraction were sufficient to accurately identify genomic clones containing sequences that are missing in the deletion mutant. We discuss the limitations and some potential applications of the method.**

Cloning the DNA corresponding to a genetic locus that is defined solely by a mutant phenotype is generally an arduous task. Such DNA can only be isolated readily from the few organisms in which transformation and complementation with genomic libraries is feasible or in which transposon or insertional mutagenesis can be utilized. Unfortunately, there are numerous mutants with interesting phenotypes in organisms for which such procedures are not yet established. A more generally applicable approach, chromosome walking, can be used in organisms amenable to linkage analysis to isolate a gene that is linked to a restriction fragment length polymorphism (RFLP) marker. Chromosome walking has the disadvantage of being laborious and can be impeded by unclonable sequences or stretches of repetitive DNA.

We describe a technique called genomic subtraction that provides a useful addition to the approaches mentioned above. The procedure identifies clones that contain sequences that are missing in a deletion mutant. It is not labor intensive and should be widely applicable, although it requires the preexistence of a homozygous deletion mutant that is viable. This paper demonstrates that genomic subtraction can be used to efficiently isolate the DNA that is absent in a yeast deletion mutant.

## MATERIALS AND METHODS

**Yeast Strains and Plasmid.** Yeast strain T1753 is $\alpha$, *ura3*, *trp1*, *his3*, *leu2*, *can*$^R$, *cir*$^0$. Strain TD33.3 is a derivative of strain T1753 that has a 5-kilobase (kb) deletion at the *lys2* locus. These strains and plasmid YIp33.3, which contains the 5-kb *Bgl* II fragment that is deleted in strain TD33.3, were provided by Dean Dawson (Tufts University School of Medicine). We confirmed the structure of the *lys2* locus in these strains by hybridizing genomic DNA digests on Southern blots with the 5-kb *Bgl* II fragment from plasmid YIp33.3 (data not shown).

**DNA.** DNA for genomic subtraction should be pure (i.e., free of DNA from biological contaminants, RNA, nucleotides, polysaccharides, and proteins). It is important that genomic DNA concentrations be measured carefully. We calculate genomic DNA concentrations by comparing band intensities after gel electrophoresis of the genomic DNA samples and several dilutions of bacteriophage $\lambda$ DNA of known concentration. Measurements of DNA concentration by absorbance spectroscopy are not generally sufficiently accurate for this application.

For the preparation of yeast DNA, pellets from 4 liters of saturated culture were resuspended in 240 ml of sorbitol at 167 mg/ml/0.2% (vol/vol) 2-mercaptoethanol/zymolase at 0.1 mg/ml ($10^5$ units/mg; Seikagaku America, Saint Petersburg, FL)/100 mM EDTA. After incubation for 1 hr at 37°C the cells were spun for 20 min at 4°C in a Beckman JS4.2 rotor at 4200 rpm. The pellets were resupended in 60 ml of 50 mM glucose/25 mM Tris·HCl, pH 8/10 mM EDTA. To the resulting 100-ml suspension we added 25 ml of 3.5 M NaCl/0.1 M EDTA/0.5 M Tris·HCl, pH 8. DNA was prepared from this suspension using a CTAB extraction procedure (1). The pellet from the final EtOH precipitation step was resuspended in 10 ml of 10 mM Tris·HCl, pH 8/1 mM EDTA (TE). RNase A (Sigma) (2) was added at 100 $\mu$g/ml and the solution was incubated at 37°C for 1 hr. The solution was brought to 0.3 M NaOAc and extracted twice with phenol/chloroform (1:1). After adding 2 volumes of EtOH, DNA was spooled out of the solution, resuspended in 5 ml of TE, and then brought to 0.3 M NaOAc. DNA was spooled out of EtOH solution twice more, washed in 100% EtOH, and resuspended in TE. A portion of the genomic DNA isolated from strain TD33.3 was sheared by sonication to an average size of about 3000 base pairs (bp) using an Ultrasonics model W-375 sonicator (Ultrasonics, Farmingdale, NY). The sheared DNA was concentrated to 0.5 ml by 2-butanol extraction, spun at 13,000 $\times$ *g* for 1 min to remove some insoluble material, denatured by boiling for 3 min, brought to 0.3 M NaOAc, precipitated by adding 2 volumes of EtOH, washed in 100% EtOH, dried, and resuspended at 1 mg/ml in $H_2O$. Tris buffer was avoided at this stage since it may react with photobiotin.

A *Sau*3A adaptor was prepared by annealing the synthetic oligonucleotides GACACTCTCGAGACATCACCGTCC and GATCGGACGGTGATGTCTCGAGAGTG. The latter oligonucleotide was phosphorylated at the 5′ end using T4

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Abbreviations: RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction.

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polynucleotide kinase (New England Biolabs) (3). An equal mass of each of the two strands was combined. The mixture was heated to 100°C for 2 min in a 200-ml water bath that was then allowed to cool to room temperature. One end of the resulting adaptor has a *Sau*3A-compatible end with a 5′ phosphate and the other end has a *Sau*3A-incompatible 5′ overhang. The incompatible overhangs and single 5′ phosphate ensure that only one adaptor molecule is ligated onto each end of a *Sau*3A fragment.

The *Bgl* II fragment from the region deleted in strain TD33.3 was subcloned by ligating a *Bgl* II digest of YIp33.3 into pUC13 that had been cut with *Bam*HI. The deleted fragment was excised from this plasmid by cutting with *Eco*RI and *Pst* I (the *Bgl* II sites had been destroyed during subcloning). This fragment, referred to as the "5-kb *Bgl* II fragment" or the "cloned deleted fragment" below, was gel purified on a 2% low-melting agarose gel (4).

**DNA Modification with Photobiotin.** Sheared denatured DNA (1 mg/ml) in $H_2O$ (see note above) was mixed with an equal volume of photobiotin acetate (2 μg/ml in $H_2O$; Clontech). Aliquots of 100 μl were incubated for 15 min in open 2-ml tubes floating in an ice bath 10 cm below a GE model RSM/H sunlamp equipped with a 275-W bulb. The reaction was brought to 100 mM Tris·HCl at pH 9 by adding a 1 M stock solution. Unreacted photobiotin was removed by extracting four times with water-saturated 1-butanol. After ethanol precipitation the modified DNA was resuspended at 2 mg/ml in 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid) (EPPS), pH 8.0/1 mM EDTA (EE). It is important to maintain a neutral pH during extensive high temperature incubations to minimize depurination of DNA (5). EPPS was used because its $pK_a$ (8.0 at 20°C) changes slowly with temperature (−0.007 unit/°C). A 1 M stock solution of EPPS at pH 8.25 was used to make all EPPS-containing buffers.

**Subtraction.** *Sau*3A-digested T1753 DNA (0.5 μg), biotinylated DNA from strain TD33.3 (10 μg), an end-labeled 84-base synthetic oligonucleotide (70,000 cpm), and 40 μg of yeast tRNA (Sigma) were denatured in 2× EE by boiling for 1 min. The 84-mer, which does not hybridize with yeast DNA, had been labeled using T4 polynucleotide kinase (New England Biolabs) and [α-$^{32}$P]ATP (New England Nuclear). The oligonucleotide molecule was included as a marker for the fractions containing DNA that did not bind to avidin and was used to estimate overall yield. The mixture was lyophilized, resuspended in 4 μl of 2.5× EE, and then mixed with 1 μl of 5 M NaCl. Hybridizations were carried out in a 65°C air incubator in 0.5-ml centrifuge tubes to maintain constant sample volumes. Since these experiments were completed we have adopted the practice of overlaying the samples with paraffin oil to prevent evaporation. After 17 hr at 65°C, 95 μl of EE/500 mM NaCl (EEN) was quickly and thoroughly mixed with the sample, which was then combined with 100 μl of a 5% suspension of avidin-coated polystyrene beads (no. 31-040-1; Pandex Division, Baxter Healthcare, Mundelein, IL) (6) that had been washed in EEN. The sample was incubated at room temperature for 30 min, added to the cup of a microcentrifuge tube filter unit (no. UFC3 OGV 00; Millipore), and spun at 13,000 × *g* for 15 sec. The beads, which were retained by the filter, were washed once with 200 μl of EEN. The unbound nucleic acid in the filtrate was precipitated at −70°C following the addition of 2 volumes of ethanol. The resulting pellet was washed with ethanol, dried, and resuspended in 5 μl of EE. We saved 0.5 μl for subsequent analysis. The sample was combined with 10 μg of biotinylated TD33.3 DNA and 20 μg of yeast tRNA and brought to 2× EE. Four more cycles of denaturation, reassociation, and avidin selection were performed as above. Aliquots (1/10 of each unbound fraction) were saved after each cycle. We added tRNA (20 μg) as needed to maintain a visible pellet after centrifugation. The recovery of DNA not capable of hybridization ranged from 80% to 90% per round, as judged by the loss of the labeled oligonucleotide. After five rounds of subtraction, the recovery of the labeled oligonucleotide was 44% of the theoretical yield.

If desired, the above protocol can be modified to permit recovery of the bound DNA. The beads are washed with EEN, the bound DNA is eluted with 100 mM NaOH, and the filtrate is neutralized.

**Addition of Adaptors.** Fragments that have melting temperature values near or below 65°C in 1 M NaCl cannot hybridize efficiently under the hybridization conditions used in these experiments and thus will be found in the unbound fraction after each round of subtraction. To ensure that such fragments were completely denatured so that they could not be capped by adaptors and thus could not be amplified, we included a high temperature incubation step prior to the addition of the adaptors. We suspended 1/5 of the DNA that was saved after cycles 1–4 and 1/10 of the unbound fraction from cycle 5 in 50 μl of 1 M NaCl/EE/tRNA at 400 μg/ml. After incubation at 80°C for 30 min, EE (200 μl) was added, the samples were precipitated and washed with EtOH, and DNA was resuspended in 5 μl of EE. Half of the DNA from each sample was ligated to *Sau*3A adaptors (50 ng) using 6 units of T4 DNA ligase (Pharmacia) in 10 μl at 15°C for 7 hr. In addition, *Sau*3A-digested T1753 DNA and the cloned, *Sau*3A-cut, gel-purified, *Bgl* II fragment were ligated to adaptors.

**DNA Amplification.** DNA capped with adaptors (1/10 of the ligation reaction) was amplified in a thermal cycler (Ericomp, San Diego, CA) with the GeneAmp kit (Perkin–Elmer/Cetus) using 0.5 μg of phosphorylated adaptor strand GACACTCTCGAGACATCACCGTCC as a primer. Primers can be phosphorylated if subsequent cloning of the amplified DNA is desired. Each of the 50 cycles included three segments: 30 sec at 93°C, 30 sec at 55°C, and 3 min at 72°C. Free nucleotides were removed from the amplified DNA by spinning samples through columns (2) composed of extensively washed Sephadex G-25 (Pharmacia).

**Colony Hybridization.** Amplified DNA (1/10 of the sample) was denatured in the presence of adaptor strand GACACTCTCGAGACATCACCGTCC (0.2 μg) and labeled using [α-$^{32}$P]dCTP and Klenow fragment of DNA polymerase I (4). Plasmid YIp33.3 containing the cloned yeast *lys2* gene was digested with *Bgl* II and *Xho* I. The two fragments comprising the *Bgl* II fragment that is deleted in strain TD33.3 were gel purified and labeled using random hexamers as primers (7). Replica filters containing about $1 \times 10^5$ yeast genomic clones were hybridized to the labeled DNA (8, 9). The yeast plasmid genomic library was constructed by Mark Rose (Princeton University).

## RESULTS

**Experimental Strategy.** The goal of genomic subtraction is to isolate wild-type DNA that corresponds to the region that is absent in a deletion mutant. The method is diagrammed in Fig. 1. An excess of sheared, biotinylated, deletion mutant DNA is denatured in the presence of a small amount of *Sau*3A-digested wild-type DNA (step A in Fig. 1). The mixture is then allowed to reassociate. Most of the wild-type strands hybridize with complementary biotinylated strands. In contrast, wild-type strands that correspond in chromosomal position to the deletion in the mutant will have no biotinylated complementary strand with which to hybridize. In the next step, the biotinylated DNA, and any DNA that has reassociated with it, are removed from the sample by incubating the reaction products with avidin-coated polystyrene beads (step B in Fig. 1). The unbound DNA, which is enriched for sequences that are missing in the deletion mutant, is collected. This fraction is now mixed with an excess of fresh biotinylated deletion mutant DNA and the

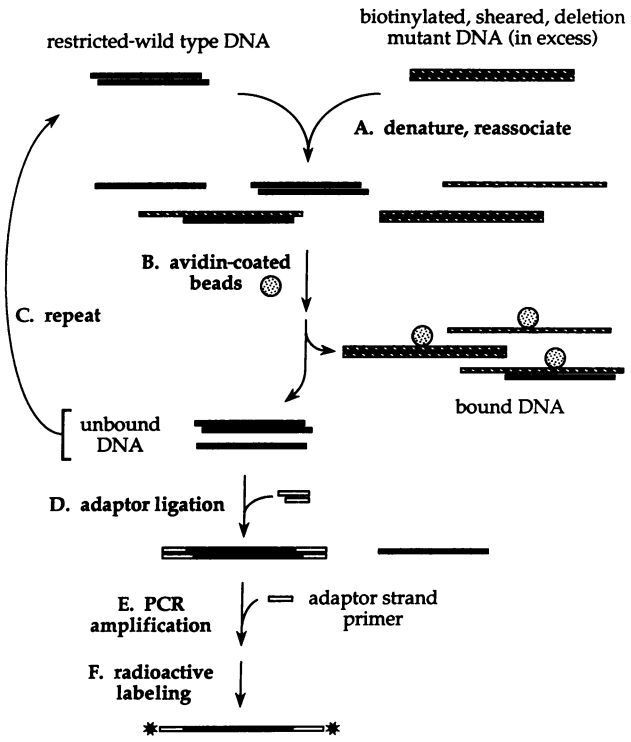


FIG. 1. Schematic representation of genomic subtraction. PCR, polymerase chain reaction.

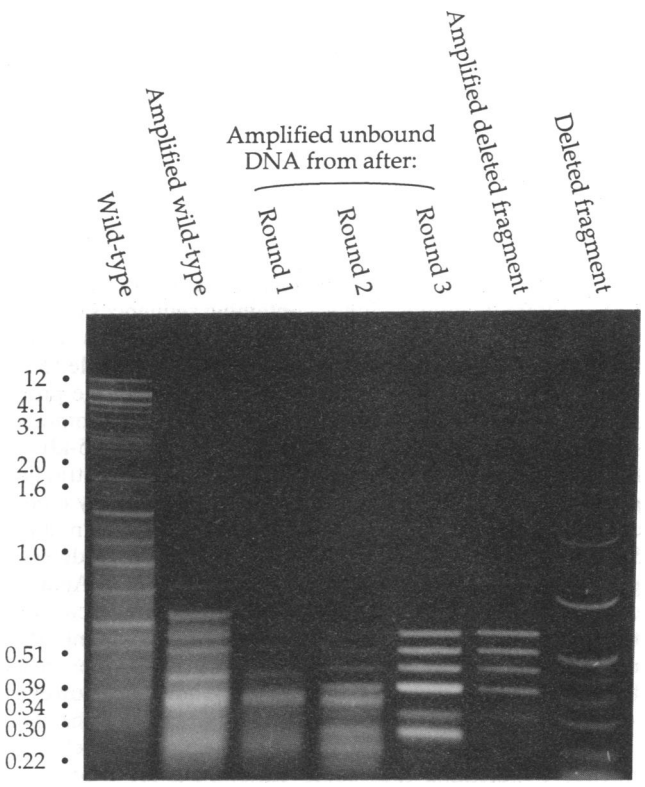


FIG. 2. Gel electrophoresis of the DNA products of genomic subtraction in 2% agarose containing ethidium bromide (0.25 $\mu$g/$\mu$l). From left to right: wild-type, T1753 DNA cut with *Sau*3A (0.75 $\mu$g); amplified wild-type, amplified *Sau*3A-digested T1753 DNA (45% of the products resulting from amplification of 50 pg of DNA); round 1–round 3, amplified unbound DNA from after the first, second, and third cycles of genomic subtraction [45% (first and second rounds) or 24% (third round) of the products resulting from the amplification of 0.1% of the unbound DNA]; amplified deleted fragment, amplified *Sau*3A digest of the 5-kb *Bgl* II fragment that is deleted in strain TD33.3 (12% of the products resulting from the amplification of 10 fg of DNA); deleted fragment, the deleted 5-kb *Bgl* II fragment digested with *Sau*3A (≈0.5 $\mu$g). Note that the first and last lanes contain fragments that were not capped with adaptors and were thus 48 bp shorter and migrated faster than comparable fragments in the other lanes. The migration of gel standards, sizes of which are expressed in kb, are indicated by the bullets on the left.

mixture is again denatured, renatured, and depleted of biotinylated sequences (step C in Fig. 1).

After several rounds of subtraction, little DNA remains in the unbound fraction. Amplification of the remaining DNA, using the PCR (10), provides enough DNA to proceed with the experiment. The PCR requires that template molecules be flanked by defined sequences that can hybridize to oligonucleotide primers. We satisfy this prerequisite by ligating oligonucleotide adaptors with *Sau*3A-compatible ends to the unbound *Sau*3A fragments (step D in Fig. 1). One strand of the oligonucleotide adaptors can then be used as a primer for the PCR since it is complementary to the 3′ end of each template molecule in the unbound fraction (step E in Fig. 1). A similar strategy for amplifying genomic sequences was reported recently (11). Radioactive nucleotides are incorporated into the amplified DNA in a final round of polymerization (step F in Fig. 1). The labeled DNA is used to screen a genomic library made from wild-type DNA. The most intense signals should correspond to colonies that contain DNA coinciding with the sequences that are deleted in the mutant.

**Genomic Subtraction Applied to Yeast.** To test the technique, we used two yeast strains that differ in that one, TD33.3, is missing a 5-kb *Bgl* II fragment covering the *lys2* gene. The deletion covers about 1/4000th of the yeast genome. DNA from wild-type strain T1753, which has an intact *lys2* gene, was digested with *Sau*3A. DNA from mutant strain TD33.3 was sheared by sonication to average size of 3000 bp. Starting with 0.5 $\mu$g of wild-type DNA and using 10 $\mu$g of biotinylated deletion mutant DNA in each round, we performed five cycles of subtraction. After each cycle we saved an aliquot of the unbound DNA onto which *Sau*3A adaptors were ligated. A fraction of each aliquot was amplified using one strand of the adaptor as a primer in the PCR. Fig. 2 shows the electrophoretic analysis of the amplified DNA derived from cycles 1–3. The products from rounds 4 and 5 looked identical to those from round 3 (data not shown). Also shown are the amplification products obtained from 10 fg of the cloned deleted *Bgl* II fragment that had been digested with *Sau*3A and then capped with adaptors. Note that the pattern of bands observed after three cycles of subtraction is nearly identical to the pattern seen upon amplification of the cloned deleted fragment. This indicates that a high degree of enrichment was achieved for the fragments corresponding to the deletion after three rounds of subtraction.

Although we obtained DNA that was highly enriched for the desired sequences, inspection of Fig. 2 indicates that the small fragments in the complex mixture of template molecules were preferentially amplified. The left-most lane in Fig. 2 shows a *Sau*3A digest of wild-type yeast DNA with bands extending as high as about 10 kb. When a small amount of the digested wild-type DNA was ligated to adaptors and amplified, products longer than about 700 bp were not observed (amplified wild-type in Fig. 2). Similarly, comparison of the two rightmost lanes in Fig. 2 indicates that the larger *Sau*3A fragments from within the cloned deleted fragment were inefficiently amplified. Note that the leftmost and rightmost lanes contain DNA that was not ligated to adaptors. Thus, fragments in these lanes migrated further than the corresponding amplified fragments in the adjacent lanes. It is possible that amplification conditions could be found that minimize the bias toward smaller products. Simply extending

the length of the polymerization step during amplification has not been effective in our hands. Digestion of the starting wild-type DNA with multiple restriction enzymes would be one way to minimize the number of inefficiently replicating large fragments.

The amplified DNA from rounds 2 and 3 was labeled and used to probe replica filters made from a plate containing about $10^5$ yeast genomic plasmid clones. In addition, the cloned 5-kb *Bgl* II fragment that is missing in strain TD33.3 was labeled and hybridized to a replica filter made from the same plate. Fig. 3 shows an autoradiogram of the three replica filters. Genomic clones containing sequences that are deleted in strain TD33.3 are indicated by the spots on the rightmost replica filter that was hybridized to the labeled 5-kb *Bgl* II-deleted fragment. After hybridization with the probe derived from the third cycle (middle filter) the prominent signals correspond to spots generated by the labeled 5-kb *Bgl* II fragment. Thus, three rounds of genomic subtraction provided sufficient enrichment to accurately identify clones containing sequences that are absent in the deletion mutant. Several of the clones that are identified by the cloned deletion probe does not appear as spots on the round 3 filter. Analysis of duplicate filters probed with the round 3 probe indicate that the spots are missing due to unfaithful replica plating (data not shown). Most of the colonies that hybridize to the probe from round 2 do not contain deleted sequences; however, several of the darker signals are superimposable on the spots on the filter that was probed with the cloned deleted sequences, indicating that some enrichment for deleted sequences has occurred after two cycles of subtraction.

We obtained results similar to those shown in Fig. 3 in two earlier genomic subtraction experiments using the same yeast strains. In one experiment, five rounds of subtraction were required to identify the correct genomic clones. In another experiment four cycles were necessary. The latter experiment was performed using an earlier protocol in which we incubated the reassociated DNA with free avidin and then bound the biotinylated molecules to a biotin-cellulose matrix (12). Analysis of cloned amplified unbound DNA from the experiment reported here indicated average enrichments of ≈10-fold during each of the first three rounds (data not shown). To be conservative, however, when designing subtraction experiments, we assume that we will achieve a 5-fold enrichment per round. For example to obtain a 10,000-fold enrichment we would perform six cycles of subtraction. One factor that may be important in determining the number of cycles needed is the extent of modification by photobiotin.

Attempts to perform genomic subtraction when adaptors were added at the beginning of the experiment (i.e., after *Sau*3A digestion of the wild-type DNA) have not resulted in enrichments equal to those shown here. This may be due to the amplification of significant amounts of DNA that remains unbound during the affinity steps for reasons other than that they correspond to a chromosomal deletion (see discussion of contaminants below).

## DISCUSSION

Using genomic subtraction we efficiently isolated sequences that are absent in a deletion that spans 1/4000th of the yeast genome. Cloning sequences that correspond to deletions that represent smaller fractions of a genome should be possible simply by performing more rounds of subtraction.

The concept of using subtractive hybridization to isolate deleted sequences was pioneered by Bautz (13), who used DNA from a bacteriophage T4 deletion mutant to isolate mRNAs from the deleted region. Several laboratories have recently published methods for enrichment of deleted DNA sequences from genomic DNA (14–18). The genomic subtraction procedure described in our paper makes it possible to achieve greater enrichments than was possible using the earlier methods.

Previous methods of enriching genomic DNA for deleted sequences fall into two classes: those that use a single round of competitive hybridization and those that use multiple rounds of subtraction. The single-step competitive hybridization approach was first used by Lamar and Palmer (14). Published enrichments using single-step methods have been <100-fold (14–16). In these procedures, an excess of sheared mutant DNA is denatured with *Sau*3A-digested wild-type DNA and then allowed to renature. The fragments that reform *Sau*3A sticky ends are then ligated to a vector and cloned. The major limitation of such methods is that the enrichment obtained can be in theory no greater than the ratio of mutant DNA to wild-type DNA. This ratio determines the fraction of the wild-type strands that reassociate with other unmodified wild-type strands in spite of the presence of complementary biotinylated mutant strands in the mixture. Another drawback of single-step enrichment protocols is the laborious final screening process in which the clones obtained must be individually analyzed to determine which ones correspond to the deletion.

Two groups have previously achieved some enrichment using methods that employ multiple rounds of subtraction (17, 18). After five rounds of subtraction Welcher *et al.* (17) achieved a 20-fold enrichment allowing identification of genomic sequences that are present in one strain of bacteria but not another. To our knowledge, these methods have been successfully applied only to cases in which substantial fractions of the genomes differ.

Genomic subtraction, though easy to perform, allows attainment of more dramatic enrichment than did previous methods. One factor critical to the success of our method is use of a matrix that binds the biotinylated DNA reproducibly

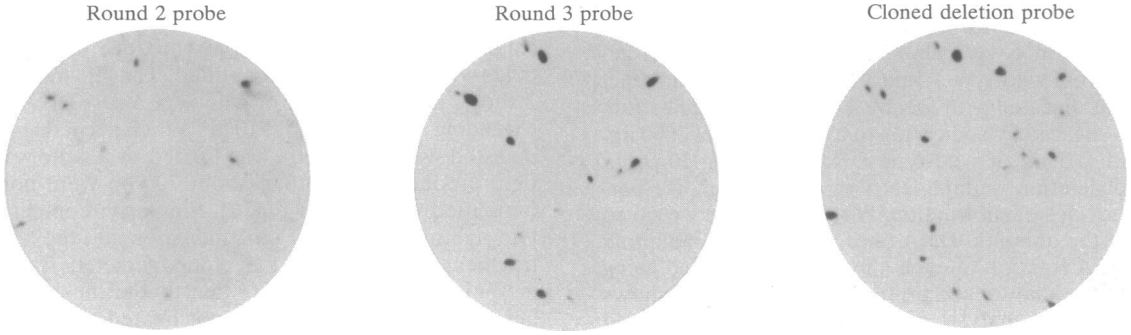


FIG. 3. Autoradiogram of three replica filters made from a plate containing about $1 \times 10^5$ yeast genomic plasmid clones. The filters were hybridized with the labeled products of the amplification of unbound DNA from after either the second (Round 2 probe) or third (Round 3 probe) subtraction cycles or with the labeled cloned DNA corresponding to the fragment deleted in strain TD33.3 (Cloned deletion probe).

and quantitatively. As discussed above, multiple rounds of subtraction are necessary to achieve high levels of enrichment. The addition of a DNA amplification step allows many cycles to be performed using small amounts of DNA while still yielding an abundance of product from which to make radioactive probe. To obtain a high degree of enrichment we believe it is critical to eliminate contamination by sequences that hybridize inefficiently. Examples of sequences that can copurify with the desired fragments are sequences with low melting temperatures that remain single stranded under the reassociation conditions and palindromic sequences that preferentially self-associate. Sequences such as these will not have the sticky ends needed for adaptor ligation and thus will not be amplified. In contrast, with methods that employ multiple rounds of subtraction without an amplification step, such sequences are represented in the final probe. Using the amplified DNA to probe a library greatly facilitates the screening process compared to analyzing individual clones derived from the enriched fragments. This screening approach permits identification of the correct genomic clones even without complete purification, since the desired sequences need only be more highly represented than any other particular sequence in the probe.

The application of genomic subtraction is subject to several constraints. Strains with homozygous or hemizygous deletions of the locus to be cloned must be available and viable. Use of overlapping deletions will minimize the likelihood of lethality due to the deletion of linked essential loci. The deletion must cover at least one restriction fragment (one *Sau*3A fragment in our experiment) that is composed entirely of nonrepetitive sequences. By digesting wild-type DNA with multiple restriction enzymes it should be possible to isolate fragments from small deletions. Ideally, the two strains should be isogenic so that sequences covered by deletions occurring at irrelevant loci are not recovered. Use of isogenic lines also minimizes the chance of isolating repetitive sequences that are more abundant in the wild-type strain than in the mutant strain.

Cloning DNA that corresponds to sequences missing in deletion mutants should be especially useful in systems in which mutants can be isolated but for which established cloning methods have not yet been developed. Because of the ease, efficiency, and speed of genomic subtraction, this method may also prove valuable in organisms in which other cloning techniques such as transposon tagging or chromosome walking are commonly used. It will be important to make and identify deletions for the method to be useful. For organisms with large genomes or those that exhibit polyteny, mutants can be screened cytologically for deletions. In the absence of cytological evidence, extensive genetic fine structure analysis is required to prove that a mutation is caused by a deletion. Rather than proving that a mutant is caused by a deficiency, genomic subtraction can be performed on a series of allelic mutants that have been induced by treatments that cause deletions at a high frequency. X-rays (19, 20), diepoxybutane (21), and chlorambucil (22), for example, can induce high proportions of deletion mutants. We have designed our method to facilitate the processing of multiple samples with a minimum of effort.

Although we have discussed genomic subtraction in the context of cloning DNA corresponding to deletions, other applications of the method may be possible. The method could also be used to isolate pathogen DNA from infected tissue. Virulence genes could be cloned from a pathogen if a related nonpathogenic strain exists. By judicious choice of hybridization conditions it may be possible to coax divergent sequences to behave as deleted sequences do in the experiments reported here. Clones obtained by applying the method to distantly related organisms could be used to probe intermediate species, allowing the construction of phylogenetic trees. Similarly, DNA fragments that are present in one strain but absent in another could be used as RFLP markers or as probes to identify unknown strains. The methodology we have developed can also be applied to experiments that require recovery of the material that is bound to the matrix. For example, a specific biotinylated fragment could be used to purify related pieces of genomic DNA that span a large region of the chromosome.

It may be possible to extend genomic subtraction to higher plants and animals by accelerating the kinetics of the DNA reassociation reactions (23). The technique could potentially be used to isolate genes that cause human diseases if patients with overlapping or hemizygous deletions can be found or if cell lines that differ in the presence or absence of the locus are available. A protocol for performing genomic subtraction can be obtained by writing to the authors.

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