MOLECULAR AND CELLULAR BIOLOGY, May 1982, p. 578–587
0270-7306/82/050578-10$02.00/0

Vol. 2, No. 5

General Method for Cloning Amplified DNA by Differential Screening with Genomic Probes

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Received 25 September 1981/Accepted 11 December 1981

Mutant Syrian hamster cell lines resistant to *N*-(phosphonacetyl)-L-aspartate, a potent and specific inhibitor of aspartate transcarbamylase, have amplified the gene coding for the multifunctional protein (CAD) that includes this activity. The average amount of DNA amplified is approximately 500 kilobases per gene copy, about 20 times the length of the CAD gene itself. A differential screening method which uses genomic DNAs as probes was developed to isolate recombinant phage containing fragments of amplified DNA. One probe was prepared by reassociating fragments of total genomic DNA from 165-28, a mutant cell line with 190 times the wild-type complement of CAD genes, until all of the sequences repeated about 200 times were annealed and then isolating the double-stranded DNA with hydroxyapatite. This DNA was highly enriched in sequences from the entire amplified region, whereas the same sequences were very rare in DNA prepared similarly from wild-type cells. After both DNAs were labeled by nick translation, highly repeated sequences were removed by hybridization to immobilized total genomic DNA from wild-type cells. A library of cloned DNA fragments from mutant 165-28 was screened with both probes, and nine independent fragments containing about 165 kilobases of amplified DNA, including the CAD gene, have been isolated so far. These cloned DNAs can be used to study the structure of the amplified region, to evaluate the nature of the amplification event, and to investigate gene expression from the amplified DNA. For example, one amplified fragment included a gene coding for a 3.8-kilobase, cytoplasmic, polyadenylated RNA which was overproduced greatly in cells resistant to *N*-(phosphonacetyl)-L-aspartate. The method for cloning amplified DNA is general and can be used to evaluate the possible involvement of gene amplification in phenomena such as drug resistance, transformation, or differentiation. DNA fragments corresponding to any region amplified about 10-fold or more can be cloned, even if no function for the region is known. The method for removing highly repetitive sequences from genomic DNA probes should also be of general use.

Mutants of mammalian cell lines have been obtained by selecting for resistance to agents which inhibit the growth of normal cells (26, 39). Resistance is often achieved through the overaccumulation of one protein or enzyme, usually the specific target of the inhibitor. In three cases, overproduction has been shown to be caused by amplification of a region of DNA that includes the gene coding for the overproduced protein. Cells resistant to methotrexate, *N*-(phosphonacetyl)-L-aspartate (PALA), or cadmium contain multiple copies of the genes for dihydrofolate reductase (20), the multifunctional protein CAD (36), or metallothionein-I (3), respectively. The high frequency with which resistant mutants have been found in about 12 other systems (26) suggests that gene amplification may be a common mechanism for attaining drug resistance. It is important to learn how widespread the phenomenon of amplification may be in different cells and in different parts of the genome.

The chromosomal location of amplified genes has been studied by cytogenetic techniques in two cases. Stable methotrexate-resistant mutant cell lines carry the amplified dihydrofolate reductase genes in an expanded region of a single chromosome (14, 25), whereas unstably resistant mutants have these genes in acentromeric chromosomal material called double minute chromosomes (20). The amplified CAD genes in Syrian hamster cells are localized in an expanded region of chromosome B-9 (38). The average amount of DNA amplified per gene copy has been estimated to be about 500 kilobases (kb) for both dihydrofolate reductase (25) and CAD (27), many times the size of the functional genes. Understanding the arrangement of amplified

units and the nature of the joints between them should help to elucidate the mechanism(s) of amplification. It is also important to study the expression of genes coamplified with the gene that was the target of the selection. We now describe the development of a general differential screening method for isolating a set of DNA fragments from amplified regions by molecular cloning and the application of this method to the CAD system. Our method uses genomic DNA probes from which highly repetitive sequences have been removed. Differential screening with cDNA probes has been used previously to clone genes differentially expressed under a variety of physiological conditions (33).

MATERIALS AND METHODS

Purification of nucleic acids. The cell lines SV28 (wild type), 165-28 (190 CAD gene copies per haploid genome), 66-6 (13 CAD gene copies per haploid genome [21, 36]), and 8-4 (100 CAD gene copies per haploid genome) were grown as described previously (34). DNA was labeled with tritium by growing the cells for 20 h in the presence of [^{3}H]thymidine (10 μCi/ml, 20 Ci/mmol).

Cytoplasmic polyadenylated [poly(A)$^+$] RNA was isolated as described previously (36). High-molecular-weight DNA was isolated by a modification of the method of Davis and Kingsbury (11). Confluent monolayers of cells were washed once with isotonic saline and lysed in situ with 10 ml of 10 mM EDTA–50 mM Tris-hydrochloride (pH 7.9)–1% sodium dodecyl sulfate (SDS) per 10-cm plate and 100 μg of proteinase K per ml. The lysate was poured into an Erlenmeyer flask and incubated at 60°C for 2 h with slow swirling. The DNA was extracted twice with 1 volume of phenol saturated with 0.5 M Tris-hydrochloride (pH 7.9) and once with chloroform before precipitation with ethanol. The DNA was suspended in 0.1 volume of TE buffer (10 mM Tris-hydrochloride [pH 7.0], 1 mM EDTA) containing 10 μg of pancreatic RNase A per ml. After treatment with proteinase K (50 μg/ml) for 1 h at 37°C in the presence of 0.1% SDS, the DNA was extracted as described above, precipitated with ethanol, and redissolved in 0.01 volume of TE buffer. Sheared DNA fragments were prepared by sonication and shown to be 150 to 500 nucleotides long by electrophoresis in an alkaline agarose gel.

Recombinant DNA techniques. The recombinant phage λ 200 and the plasmids pCAD41 and p102 have been described (27, 36). Phage λ 102 (a gift from G. Wahl) was constructed by inserting genomic fragment 102 into phage vector Charon 27 (4). Charon 4A (4) and the recombinant phages were grown in *Escherichia coli* strain LE392 and purified as described by Davis et al. (12). Phage DNAs were isolated as described by Maniatis et al. (23). A library of 165-28 DNA fragments in Charon 4A was constructed by the method of Maniatis et al. (23) with modifications. To prepare the arms of Charon 4A, the cohesive ends of the phage DNA (400 μg/ml) were first ligated with T4 DNA ligase. The internal fragments were then excised by digestion with *Eco*RI endonuclease (a gift of J. Carlson) and separated from the ligated arms on a 10 to 40% sucrose gradient containing 20 mM Tris-hydrochloride (pH 7.9), 1 mM EDTA, 200 mM NaCl, and 5 μg of ethidium bromide per ml, which permitted the DNA bands to be seen under UV light. The gradient was centrifuged for 24 h at 27,000 rpm and 20°C. The ligated arms were collected with a syringe and precipitated with ethanol. DNA (100 μg) from 165-28 cells was digested with a limited amount of restriction endonuclease *Eco*RI, the amount chosen to just give complete digestion in 1 h. Portions of samples withdrawn during the course of the reaction were analyzed by electrophoresis in a 0.5% agarose gel. Five samples, ranging from almost undigested to completely digested DNA, were pooled, and the 15- to 20-kb-long fragments were isolated by centrifugation through a sucrose gradient under the conditions described above for preparing the arms of Charon 4A, but without ethidium bromide. The recombinant phage DNAs were packaged in vitro as described by Enquist and Sternberg (15). We found that adding the freeze-thaw lysate in ten equal portions at 5-min intervals during the course of the reaction increased the efficiency of packaging 10-fold. Hybridization to phage plaques in situ was done as described by Davis et al. (12).

Preparation of probe DNAs for differential screening. Probes were prepared in pairs with genomic DNA from wild-type or mutant cells. Sheared ^{3}H-labeled DNA (0.1 mg/ml for SV28 and 165-28, 1 mg/ml for SV28 and 66-6) was denatured by being boiled and incubated at 65°C in the presence of 0.12 M sodium phosphate (pH 6.8) until a C_0t value of 1 (SV28 and 165-28) or 10 (SV28 and 66-6) was reached. C_0t is the product of the initial concentration of DNA in moles of nucleotide per liter and the time in seconds (6). The renatured repetitive DNA was removed by binding it to hydroxyapatite at 60°C in 0.12 M phosphate buffer (5). The single-stranded DNA was dialyzed against a buffer containing 10 mM Tris-hydrochloride (pH 7.9), 100 mM NaCl, and 1 mM EDTA, precipitated with ethanol, suspended in 0.12 M phosphate buffer at a concentration of 0.5 mg/ml (SV28 and 165-28) or 1.5 mg/ml (SV28 and 66-6), and incubated at 65°C until a C_0t value of 100 (SV28 and 165-28) or 300 (SV28 and 66-6) was reached. The renatured DNA was isolated by binding it to hydroxyapatite, dialyzed, and precipitated with ethanol.

Nick translation. DNAs were labeled with ^{32}P by the method of Rigby et al. (31). Each reaction was continued until the specific activity reached at least 10^8 cpm/μg. Unincorporated nucleotides were removed by chromatography on a 1-ml column of Sephadex G-75 equilibrated in TE buffer. The probes were then incubated for 1 h at 37°C in the presence of 0.1% SDS and 50 μg of proteinase K per ml. This treatment prevented the high backgrounds sometimes observed in hybridization experiments. We also observed that background hybridization can sometimes be removed from filters by treating them with 0.1% SDS and 50 μg of proteinase K per ml in TE buffer for several hours at 37°C.

Removal of highly repeated sequences from the genomic DNA probes used in differential screening. High-molecular-weight denatured DNA from SV28 cells was bound to diazonium cellulose as described by Noyes and Stark (24). The DNA (2.5 mg/ml in 500 mM sodium acetate buffer, pH 4) was boiled for 2 min and diluted with 4 volumes of dimethyl sulfoxide. Freshly

prepared diazonium cellulose (100 mg/mg of DNA) was added, and the mixture was incubated for 20 h at room temperature. Noncovalently bound DNA was removed by washing the cellulose once with water, once with 100 mM NaOH–10 mM EDTA, and several times with TE buffer. The product (8 μg of DNA/mg of cellulose) was incubated for 2 h at 42°C before hybridization in 50% formamide, 0.75 M NaCl, 50 mM sodium phosphate buffer (pH 6.8), 5 mM EDTA, 0.1% SDS, 5× Denhardt reagent (1× Denhardt reagent contains 0.02% [wt/vol] each bovine serum albumin, polyvinylpyrrolidone, and Ficoll [M_r 400,000] [13]), 100 μg of sonicated denatured salmon sperm DNA per ml, and 1% (wt/vol) glycine. ^{32}P-labeled denatured probe (1 μg) was incubated for 48 h at 32°C in the presence of 500 μg of immobilized DNA in 4 ml of the same mixture without glycine and with 1× instead of 5× Denhardt reagent. Every 12 h the DNA-cellulose was pelleted by centrifugation, and the probe in the supernatant solution was melted by heating for 10 min at 80°C and then added back to the same DNA-cellulose for continued incubation at 32°C. The DNA-cellulose can be reused after a wash with 100 mM NaOH–10 mM EDTA for 10 min at room temperature followed by several washes with TE buffer. It was stored in TE buffer at 4°C.

Removal of highly repeated sequences from the probes used to detect RNA or DNA after transfer to paper. Two different methods were used to remove highly repeated sequences from the probes. (i) DNA-cellulose method: phage DNAs labeled by nick translation were denatured and incubated with immobilized denatured SV28 DNA as described above for the genomic DNA probe. (ii) Hydroxyapatite method: labeled phage DNAs were mixed with a 600-fold excess of sheared SV28 DNA, corresponding to 1% of the unique sequences present in the phage DNA. The DNAs were denatured by boiling for 5 min and incubated in 0.12 M sodium phosphate buffer (pH 6.8) at 65°C until a C_0t value of 3 was reached for the SV28 DNA. This value was high enough to allow the highly repetitive sequences with a $C_0t_{1/2}$ of 0.01 to reanneal. The phage DNA remained single stranded because it reached only C_0t 0.005, one tenth of its $C_0t_{1/2}$. Double-stranded DNA was removed with hydroxyapatite, and the single-stranded fraction was used as the probe.

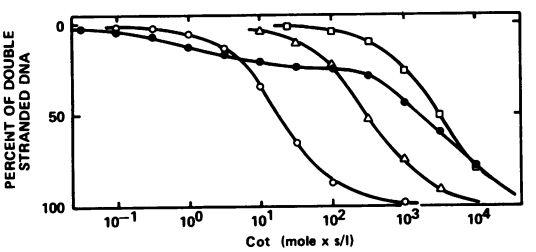

FIG. 1. Kinetics of reassociation of a fragment of the CAD gene with DNA from wild-type or PALA^r cells. Fragment 102 (27), excised from p102 DNA by digestion with *Bam*HI restriction enzyme, was purified by electrophoresis in an agarose gel and labeled by nick translation to specific activity 1.4×10^8 cpm/μg. Reactions were carried out with excess SV28 (○), 66-6 (△), or 165-28 (□) DNA. The reaction mixtures contained unlabeled cellular DNA (800 μg in 0.1 ml and 10 or 1,300 μg in 1 ml), 500 pg of the labeled fragment, and 3 μg of tritiated cellular DNA (24,000 to 80,000 cpm/μg). They were heated at 100°C for 5 min, and sodium phosphate buffer (pH 6.8) was added to a final concentration of 0.12 or 0.40 M in the 1 and 0.1 ml mixtures, respectively. After a zero-time sample was taken, the mixtures were covered with mineral oil and incubated at 65°C. Portions were removed at appropriate times, and the extent of reassociation was determined by chromatography on hydroxyapatite (5). C_0t values were corrected to a sodium phosphate concentration of 0.12 M (5). The reassociation kinetics of the total tritiated DNAs of SV28, 66-6, and 165-28 were almost identical, and only the curve for 165-28 DNA was plotted (●).

Gel electrophoresis, transfer to paper, and hybridization. DNA fragments or glyoxal-treated RNAs were separated by electrophoresis in agarose gels, stained with ethidium bromide, and transferred to paper as described by Wahl et al. (36, 37), except that aminothiophenol paper was used (Brian Seed, personal communication) and the transfers were performed in 200 mM sodium acetate buffer (pH 4). Hybridization of the purified probes to DNA or RNA immobilized on paper was done by the method of Wahl et al. (36) with slight modifications. Filters were prehybridized for at least 4 h and hybridized for 20 h at 42°C. They were washed twice for 15 min each at room temperature in 0.3 M NaCl–2 mM EDTA–20 mM sodium phosphate (pH 6.8)–0.1% SDS and twice for 30 min each at 70°C in 15 mM NaCl–0.1 mM EDTA–1 mM sodium phosphate (pH 6.8)–0.1% SDS.

RESULTS

Differential screening with genomic DNA probes. The rate of association of a given DNA sequence is proportional to its degree of repetition in the genome (6): sequences unique in one cell type and amplified in another renature at different rates. A labeled probe corresponding to part of the CAD gene and free of repetitive sequences was reannealed in the presence of excess DNA from SV28 cells (wild type) or 165-28 cells (190 copies of the CAD gene per haploid genome) (Fig. 1). In the presence of wild-type DNA the probe renatured with the unique sequences ($C_0t_{1/2}$, 3,000), whereas in the presence of 165-28 DNA it renatured about 190 times faster ($C_0t_{1/2}$, 16). The fraction of 165-28 DNA reassociating between C_0t 1 and C_0t 100 (15% of the total) included 80% of any sequence repeated about 200 times per haploid genome, but only 5% of any unique sequence. Therefore, such a repeated sequence will be $(190 \times 0.8)/(1 \times 0.05) = 3{,}000$ times more concentrated in the C_0t 1 to C_0t 100 fraction from mutant DNA than in this fraction from wild-type DNA. These two fractions can be used as differential probes to reveal recombinant phage containing amplified DNA because the signal with the probe derived from mutant DNA will be much stronger. A phage containing genomic DNA repeated equally in

wild-type and mutant cells (ribosomal genes, histone genes, etc.) should give comparable signals with both probes.

To test the differential screening method, we prepared the C_0t 1 to C_0t 100 fractions from SV28 and 165-28 DNAs and used them as probes in a control experiment with recombinant phage λ 102, which contains an 8.6-kb fragment of the CAD gene and is free of repetitive sequences (27, 28). Only the probe derived from 165-28 DNA revealed the plaques (Fig. 2A). When the same probes were used with recombinant phage λ 200, which contains a 13-kb DNA fragment of the CAD gene and includes several copies of a highly repeated sequence (27, 28), a strong signal was observed with both probes (Fig. 2A). The highly repetitive DNA in the probes hybridized to similar sequences in the cloned cellular DNA, obscuring the differential signal. During the preparation of the probes, most of the highly repetitive sequences reassociated before C_0t 1 and were retained on hydroxyapatite; nevertheless, the C_0t 1 to C_0t 100 fraction still contained some of these sequences. It was necessary to remove them from the probe in order to observe the differential signal. We accomplished this by annealing the labeled probe DNAs to total high-molecular-weight SV28 DNA immobilized on diazonium cellulose. During this step the stringency of the annealing reaction was reduced by lowering the temperature to 32°C. As the plaque hybridizations were carried out at 42°C, any repetitive sequence that was unable to anneal during the purification step should be unable to hybridize during the screening. It was important that the DNA bound to the cellulose was not extensively sheared; immobilized sonicated DNA did not remove the highly repetitive sequences from the probes, possibly because the average length of homologous sequences shared between the sonicated DNA (150 to 500 nucleotides) and the probes (20 to 150 nucleotides) was too short to permit the hybrids to be stable. Under the hybridization conditions stated, 30 to 40% of the radioactivity of the C_0t 1 to C_0t 100 fractions was retained on the DNA-cellulose. It was also possible to use high-molecular-weight SV28 DNA bound to diazonium paper (19) instead of cellulose to remove the highly repetitive sequences from the probes, but manipulations with the paper were less convenient. Attempts to remove these sequences during hybridization by competition with excess total SV28 DNA or partially purified highly repetitive sequences (C_0t 0 to C_0t 1) gave poor, irreproducible results.

When genomic DNA probes from 165-28 or SV28 were purified by this method and tested with λ 200, a clear differential signal was obtained (Fig. 2A), indicating that it would be possible to screen for phage containing a fragment of amplified DNA with a probe prepared from the DNA of a mutant containing about 200 gene copies per haploid genome. However, it is not likely that this degree of amplification can be obtained very often in other cases. Consequently, we repeated the control screening experiments with probes prepared from the DNAs of SV28 and mutant 66-6, which has only a 13-fold increase in the number of CAD genes. In this case the C_0t 10 to C_0t 300 fraction was used (see Fig. 1). This fraction (15% of the total DNA) contained 50% of any sequence repeated about 10 times per haploid genome and 10% of unique DNA, resulting in a relative enrichment of 65-fold for the amplified sequences. A total of 15% of the cellular DNA reassociated between C_0t 10 and C_0t 300. The probes from 66-6 and SV28 still gave a differential signal with λ 102, whether or not highly repetitive sequences (10% of the C_0t 10 to C_0t 300 fraction) were removed with DNA cellulose (Fig. 2B). After removal of the highly repetitive sequences, a differential signal was also obtained with λ 200. The differential method was capable of detecting phage containing fragments from regions amplified as little as 13-fold and may be useful when the degree of amplification is as low as 5- to 10-fold. Of course, the frequency in the library of such phage will be much lower than the frequency of phage from regions amplified 200-fold. The high general background in Fig. 2B was related to the high concentration of probe DNA needed in the experiments. A high concentration was needed because the amplified sequences complementary to λ 102 or λ 200 DNAs in this fraction of DNA from mutant 66-6 represented only 0.02% of the probe. The background was not eliminated by prehybridizing the filters overnight instead of for 2 h or by washing them at 70°C instead of at 50°C; it was reduced by using a concentration of probe 5 times lower, but then the signal was too low.

Molecular cloning of amplified DNA fragments. The library of genomic DNA fragments from 165-28 cells contained 7×10^5 independent recombinants, enough to contain the whole genome in 15-kb pieces with a probability of 97% (8). Forty thousand recombinant phage were screened differentially with the C_0t 1 to C_0t 100 fractions from SV28 or 165-28 DNAs used as probes. Of the plaques, 104 appeared to give differential signals (Fig. 3A), some much clearer than others. In this first screening we picked any plaque which displayed even a slight difference in intensity. Plating at low density (10 to 30 plaques per cm^2) and having many plaques which hybridized with both probes made it easy to align the plate with the autoradiogram. In most cases, the single plaque which responded differentially could be picked cleanly. Each was

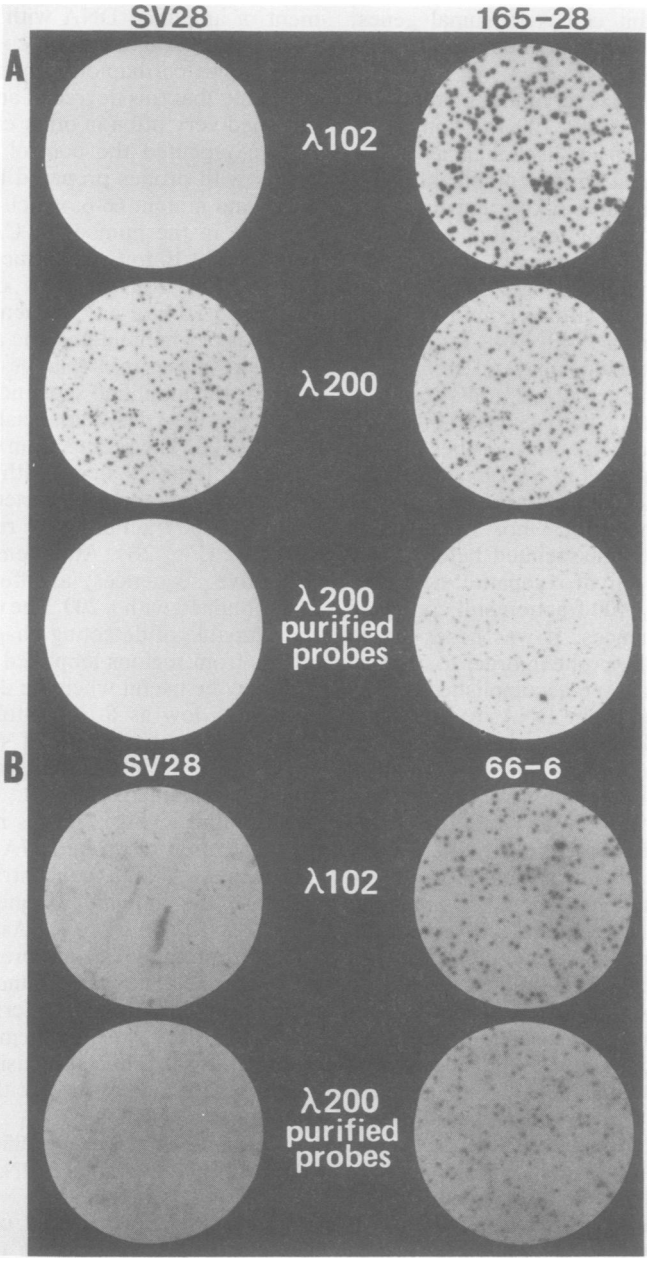

FIG. 2. Differential hybridization with probes from genomic DNAs of wild-type or PALA^r cell lines. Probes were prepared (A) from SV28 and 165-28 DNAs (C_0t 1 to C_0t 100 fraction, 1.3×10^8 cpm/μg) or (B) from SV28 and 66-6 DNAs (C_0t 10 to C_0t 300 fraction, 10^8 cpm/μg), and repetitive sequences were removed by hybridization to DNA cellulose. Replica filters (8-cm diameter) prepared from plates containing phage λ 102 or λ 200 were hybridized (A) for 20 h or (B) for 40 h at 42°C with 1 ml of unpurified or purified probes derived from wild-type or mutant DNA. The concentration of the probes was (A) 50 ng/ml or (B) 250 ng/ml, and the filters were exposed for (A) 20 h or (B) 40 h.

then replated at 5 to 10 plaques per cm^2 and screened differentially again. Only 27 (25%) gave a differential signal in the second screening (Fig. 3B); they were the ones that had given a clear differential signal the first time. One isolated plaque was picked for each clone. After the second screening the clones were usually pure. In later experiments we picked only those

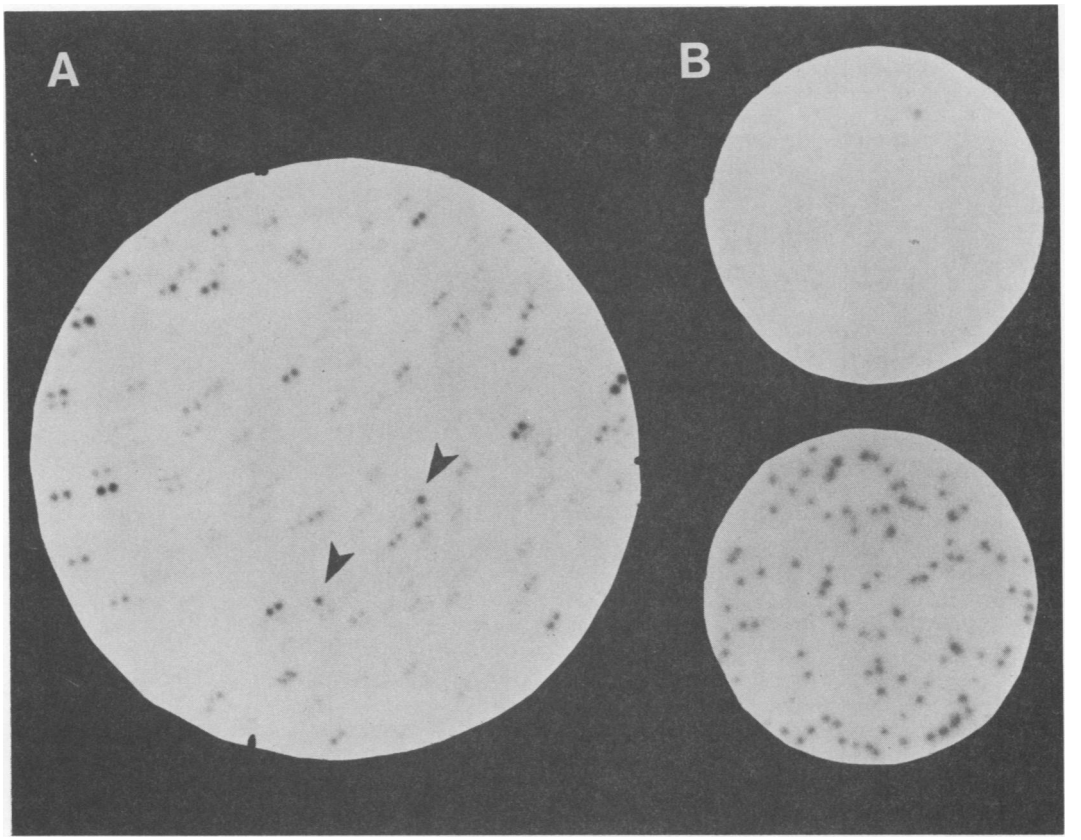

FIG. 3. Differential screening of a library of genomic DNA fragments from the PALA^r cell line 165-28. (A) Samples of the library were plated (500 phage per 100-mm plate), and replica filters were hybridized with each probe (1 ml per filter, 50 ng/ml, 9×10^7 cpm/μg) for 20 h at 42°C. The filters were washed and then autoradiographed for 15 h. The two autoradiograms were superimposed, with a slight displacement. Plaques which did not hybridize differentially are seen as doublets while those that did are seen only with the probe derived from 165-28 DNA (arrows). (B) One plaque which hybridized differentially was picked, and the phage were suspended in buffer. A portion corresponding to 100 phage was plated on one 50-mm plate, and replica filters were hybridized and autoradiographed as described above. Top plate: probe derived from SV28 DNA; bottom plate: probe derived from 165-28 DNA.

plaques hybridizing in a clear differential manner in the first screening. More than 80% of these behaved the same way in the second screening.

All of the cloned hamster DNA fragments isolated by the differential screening procedure were analyzed by restriction endonuclease site mapping and were found to fall into nine independent groups containing a total of 165 kb of amplified sequences. Of these 165 kb, 65 kb corresponded to the CAD gene and to DNA sequences at both the 5′ and the 3′ ends of the gene. The organization of the remaining 100 kb of cloned amplified DNA has yet to be determined.

Hybridization of cloned amplified fragments to cellular RNA and DNA. To provide definitive evidence that the cloned fragments we had isolated did contain the DNA present in mutant cells in much greater abundance than that in wild-type cells, we used the DNA of the phage to probe transfers to paper of SV28 or 165-28 DNA digested with *Eco*RI endonuclease. All of the recombinant phage isolated contained at least one copy of highly repeated sequences which had to be removed before probing by using either hydroxyapatite or DNA-cellulose. With most of the recombinant phage, the hydroxyapatite method gave a level of repetitive sequences low enough to allow the observation of fragments present once per haploid genome. With the remaining phage, the probes had to be purified by the more tedious DNA-cellulose method in order to achieve the same sensitivity. The results of a transfer-hybridization experiment are shown in Fig. 4A. The two fragments of cellular DNA (12 kb and 3.9 kb) present in the DNA of phage λ 5-800 (lane 1) were also present in the DNA of wild-type (lane 2) or mutant (lane

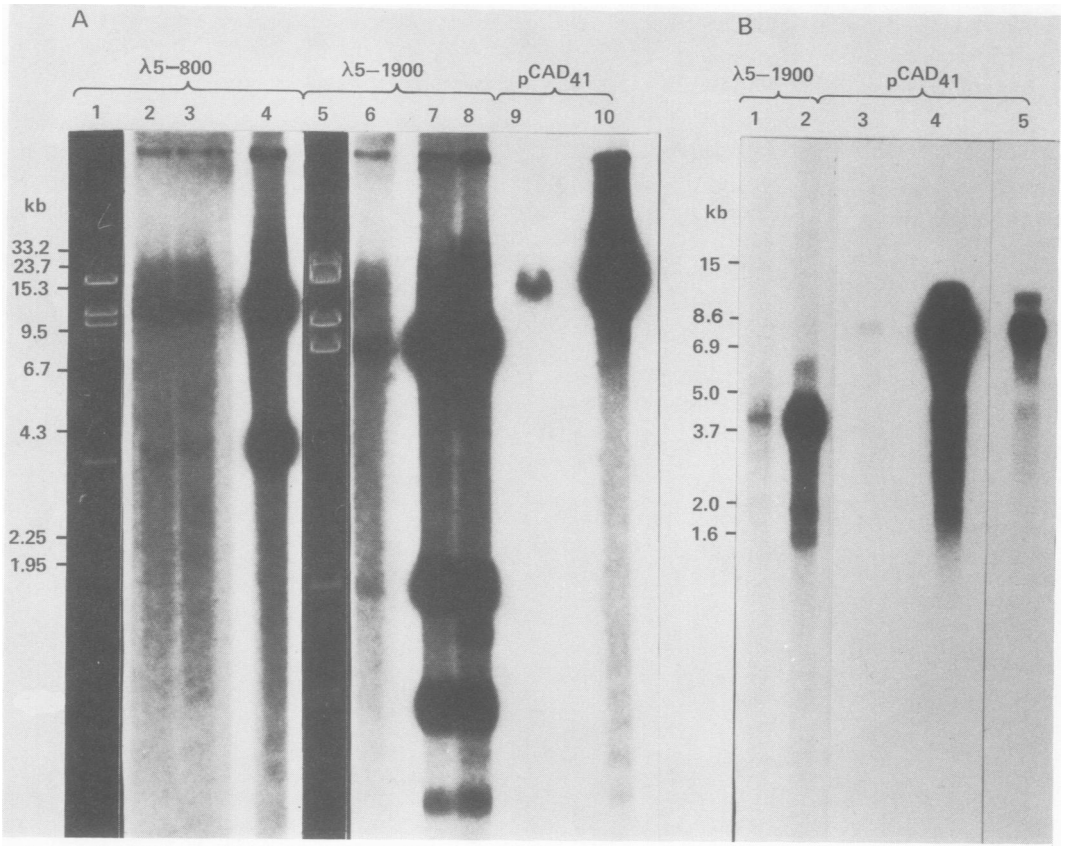

FIG. 4. Hybridization of cloned amplified fragments to cellular DNA and poly(A)$^+$ RNA. (A) DNA (1 μg) from phage λ 5-800 (lane 1) or λ 5-1900 (lane 5) or 10 μg of DNA from cell lines 165-28 (lanes 4, 8, and 10), B8-4 (lanes 3 and 7), or SV28 (lanes 2, 6, and 9) were digested with *Eco*RI endonuclease. The fragments were separated by electrophoresis in a 0.7% agarose gel. After transfer, the filters were hybridized for 20 h with λ 5-800, λ 5-1900, or pCAD41 DNA probes (1×10^8 to 1.5×10^8 cpm/μg). The two phage DNA probes were purified by the DNA-cellulose method. Lanes 1 and 5: ethidium bromide staining. Other lanes: autoradiograms exposed for 5 days. Size markers were phage λ DNA digested with *Sal*I or *Hin*dIII endonuclease. (B) Cytoplasmic poly(A)$^+$ RNA (10 μg) from cell lines SV28 (lanes 1 and 3) or 165-28 (lanes 2, 4, and 5) was treated with glyoxal and subjected to electrophoresis in a 0.8% agarose gel. After transfer, the filters were hybridized for 20 h with λ 5-1900 or pCAD41 DNA probes. The phage DNA probe was purified by the DNA-cellulose method. Autoradiograms were exposed for 4 days, except for lane 5, which is the same as lane 4 but exposed for 20 h. Size markers were phage λ DNA digested with *Ava*I endonuclease and ribosomal 18S and 28S RNAs from SV28 cells; all of these were treated with glyoxal.

4) cells. Clearly, these fragments were amplified in the mutants. From the relative intensities of the bands in lane 2 and 4 and from comparison of these with the signals obtained for the CAD gene itself (lanes 9 and 10), we conclude that these fragments were amplified to about the same extent as the CAD gene, i.e., about 200-fold. A fragment that lies wholly within the amplified region should be present in restriction endonuclease digests of both wild-type and mutant DNAs, but will be more abundant in the mutant. All of the phage analyzed contained cloned *Eco*RI fragments that were the same size as the homologous fragment of cellular DNA, indicating that no significant deletions occurred during cloning. In some cases, additional bands were observed in the 165-28 DNA (Fig. 4A, lane 8). The 1.2-kb fragment in 165-28 DNA was not present in the DNA of phage λ 5-1900 (lane 5) or in the DNA of wild-type cells (lane 6). Cases such as this are interesting because a cloned fragment that includes a joint between two units of amplified DNA should hybridize to a novel amplified fragment in digests of this DNA, as the joint is not present in the DNA of wild-type cells. The relatively low intensity of the novel band (Fig. 4A, lane 8) might be due to a short region of homology between the fragment and the probe, or it might be a consequence of the complexity of the amplified DNA in mutant 165-

28, which was selected in four steps to a high level of resistance.

The cloned fragments can also be used to reveal whether each one is always amplified in independently derived mutants. The two cellular DNA fragments contained in λ 5-800 DNA were amplified in mutant 165-28 (Fig. 4A, lane 4) but not in mutant B8-4 (lane 3), which has a 100-fold amplification of the CAD gene. On the other hand, the five fragments cloned in λ 5-1900 (8.5, 1.8, 0.9, 0.82, and 0.33 kb) were amplified in both mutants 165-28 (lane 8) and B8-4 (lane 7). It is also clear that the additional 1.2-kb fragment seen in 165-28 DNA (lane 8) was not present in B8-4 DNA (lane 7). Although they were not visible in this experiment, the 0.82- and 0.9-kb fragments were seen in the wild-type DNA in another experiment, but the signal was not strong enough for photographic reproduction. Due to the weakness of the signal, we do not know if the 0.33-kb fragment is present in wild-type DNA. A detailed analysis of the structure of the amplified DNA in different mutants is in progress and will be presented elsewhere.

Finally, we used the phage DNAs from each of the nine independent groups to probe transfers to paper of cytoplasmic poly(A)$^+$ RNAs from wild-type and mutant cells to determine whether transcriptional units other than the one for the CAD gene were amplified. The phage λ 5-1900 which has been mapped adjacent to the 5′ end of the CAD gene contained at least part of a gene coding for a 3.8-kb poly(A)$^+$ RNA which was present in wild-type cells (Fig. 4B, lane 1) and overproduced to the same extent as the two CAD mRNAs (7.9 and 10.2 kb) in mutant cells (compare lanes 1 and 2 with 3 and 4). The function of this RNA is unknown, but it cannot code for any of the last three enzymes of the pyrimidine pathway as their activities are not elevated in PALA^r cells (21). Thus, of the 165 kb of cloned DNA from the amplified region, the 65 kb contiguous to and including the CAD gene have been shown to encode five poly(A)$^+$ RNA species: the 7.0- and 10.2-kb CAD mRNAs, the 3.8-kb RNA mentioned above, and the 0.8- and 1-kb RNAs described by Padgett et al. (27). There were no poly(A)$^+$ RNA species hybridizing to the remaining 100 kb of cloned amplified DNA sequences.

DISCUSSION

The method described here for isolating cloned fragments of amplified DNA can be used even if the degree of amplification is only about 10-fold. As the method does not rely on any function of the DNA to be cloned, it can be especially useful when gene amplification is suspected but no known protein is overproduced, as for example in the case of multidrug cross-resistance in mutants of neuroblastoma cells (2). The method can also be used to evaluate the possible involvement of gene amplification in phenomena such as transformation or differentiation. For example, double minute chromosomes, observed in many types of transformed cells (1, 29, 30), were shown recently to contain amplified DNA in mouse adrenocortical tumor cells (18), and Pall (29) has proposed that gene amplification may be a cause of carcinogenesis.

During the development of an organism, some gene products are needed in large quantities only at specific stages. The required high level of expression is sometimes reached through amplification of the corresponding genes; ribosomal genes are amplified in *Xenopus laevis* oocytes (7, 16, 17), and the genes for chorion proteins are amplified during oogenesis in *Drosophila melanogaster* (32). Terminal differentiation leads to the appearance of highly specialized cells which synthesize large quantities of a small number of specific proteins, and it may be speculated that at least in some cases gene amplification might be involved. Our method can be used to prepare probes from the DNAs of cells at two different stages of differentiation or from two different organs, and these can be used to search for fragments of amplified regions cloned from the DNAs of these cells or organs.

George and Powers (18) have isolated fragments of the amplified DNA of mouse adrenocortical tumor cells by picking random clones from a library prepared from the DNA of purified double minute chromosomes. The method described here is more general because the chromosomal location of the amplified sequences does not need to be known and no fractionation of these sequences is required. However, the differential screening method should be even more powerful when applied to a library constructed by the method of George and Powers or one constructed from the DNA of chromosomes purified by chromosome sorting (22). In such cases, the frequency in the library of clones containing amplified DNA should be very high, which would facilitate screening when the degree of amplification is low.

The library prepared from the DNA of 165-28 cells had a surprisingly low frequency of clones responding differentially, 27 of 40,000 or 0.07%. Cytogenetic evidence indicates that about 500 kb of DNA are amplified for each 25-kb CAD gene in other PALA^r mutants (38); therefore, the frequency of clones carrying a fragment of amplified DNA was expected to be (500 kb/gene × 190 genes/haploid genome)/(3×10^6 kb/haploid genome) = 3%. The low frequency was probably related to preparation of the cellular DNA fragments by partial digestion with endonuclease *Eco*RI. In more recent experiments, the fre-

quency of clones containing sequences from the CAD gene was as expected when the genomic DNA fragments were prepared by mechanical shear (W. Liao and G. Stark, unpublished data).

We have devised effective procedures for removing highly repetitive sequences from genomic DNA probes. These methods can be of general use in solving the nagging problem of unwanted hybridization to the ubiquitous highly repetitive sequences present in most fragments of genomic DNA larger than 5 kb (10), in nuclear RNAs (9), and in many cytoplasmic poly(A)$^+$ RNAs (35). Removing the repetitive sequences from the fragmented probe avoids the more tedious job of finding a segment devoid of highly repetitive sequences in a cloned piece of DNA and purifying it before use as a probe.

One fragment of DNA coamplified with the CAD gene contained sequences complementary to a 3.8-kb poly(A)$^+$ RNA which is overproduced to a degree similar to that of CAD mRNA in PALA^r mutant cells. This is the third "gene" coamplified with CAD in these cells; Padgett et al. (27) have described two smaller poly(A)$^+$ RNAs (0.8 and 1 kb) of unknown function which are transcribed from DNA just beyond the 5′ end of the CAD gene and which are also overproduced in PALA^r cells. Preliminary results indicate that the gene coding for the 3.8-kb RNA is localized in the same region, about 10 kb further away from the CAD gene. The level of expression of the four genes described here was roughly proportional to their copy number. This was also true for all cases in which the overproduction of the protein target of a selection procedure was due to gene amplification. Of course, the expression of some other coamplified genes may well be regulated at normal levels. If such cases can be found, it will be very interesting to study the regulatory mechanisms as so little is known now about the control of gene expression in higher eucaryotes.

ACKNOWLEDGMENTS

We thank Janine Zieg and Miles Brennan for many helpful discussions and Richard Padgett, Geoffrey Wahl, John Carlson, and Carl Mann for gifts of materials.

This investigation was supported by Public Health Service grant CA-17287 from the National Cancer Institute. O.B. is an Attaché de Recherche of the Centre National de la Recherche Scientifique (CNRS) and was supported by fellowships from NATO and from the National Institutes of Health–CNRS Program for Scientific Collaboration. F.A. is the recipient of junior fellowship no. J-1-81 from the American Cancer Society, California Division.

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