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Genetics

Efficient cloning of single-copy genes using specialized cosmid vectors: Isolation of mutant dihydrofolate reductase genes

(*Bgl* I/recombinant DNA methods/CHO cells/gene transfer)

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ABSTRACT **A method for the efficient cloning of single-copy genes from restriction digests of mammalian DNA is described. The method is illustrated by the cloning of several mutant genes as well as the wild-type gene for Chinese hamster dihydrofolate reductase (DHFR; 7,8-dihydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3). This gene is isolated within a 41-kilobase *Bgl* I fragment by using cosmid (plasmids containing a cohesive-end site) vectors that have been constructed especially for this purpose. Two cosmids are used: one contains a short region from the 5′ flanking region of the *dhfr* gene, and the other contains a short region from the 3′ flanking region. These two regions contain the *Bgl* I sites that bound the *dhfr* gene. *Bgl* I leaves staggered ends that are different depending on the DNA sequence within the enzyme binding site. When these cosmids are cut with *Bgl* I and hybridized with total *Bgl* I-cut genomic DNA, they preferentially associate with the fragment bearing the *dhfr* gene, since it has the same *Bgl* I ends. An ≈500-fold enrichment for the *dhfr* gene in cosmid libraries from Chinese hamster ovary cells was achieved by using this method coupled with a single-step size fractionation. As a result, only several hundred cosmid colonies need to be screened in order to clone a *dhfr* gene from a particular mutant Chinese hamster ovary cell. This method should facilitate the repetitive cloning of any gene or gene fragment.**

The cloning of "single-copy" genes from extremely complex DNA mixtures such as that represented by mammalian genomes can be accomplished by using a variety of plasmid or bacteriophage vectors. For each gene isolation, genomic libraries large enough to contain at least one copy of the gene in question must be constructed and then screened with an appropriate hybridization probe. The size of these libraries is minimized by using vectors capable of harboring large inserts. At present, cosmids [plasmids containing a cohesive-end site (*cos* site)] packaged in bacteriophage λ particles represent the vehicles capable of carrying the largest amount of eukaryotic DNA, 45 kilobases (kb) (1, 2). Even with this relatively large stretch of DNA, several hundred thousand cosmid colonies must be screened in order to be reasonably sure of isolating a desired single-copy sequence. This task is not so formidable if the goal is to isolate a new gene and characterize its structure. However, in some genetic approaches, the same locus must be analyzed repeatedly for novel structural changes (e.g., mutations, recombinations). For instance, in the most extensive study of this type in mammals, the analysis of mutations at the human β-globin locus, more than 30 mutant alleles have been cloned (for review, see ref. 3). In an approach of this type, the cloning of the mutant genes can be a rate-limiting step in the genetic analysis.

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In our own studies of mutation at the dihydrofolate reductase (*dhfr*) locus in Chinese hamster ovary (CHO) cells, we were faced with the problem of isolating large numbers of mutant *dhfr* genes from a collection of dihydrofolate reductase (DHFR; 7,8-dihydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3)-deficient mutants (4, 5). Given the large size of the *dhfr* gene (26 kb; ref. 6), it was logical to use cosmid vectors so that the mutant genes could be cloned in one piece. However, we sought ways to minimize the number of cosmid colonies that would have to be screened.

Seed (7) has described an ingenious method for the specific isolation of single-copy genes. In this system (known as πvx), libraries constructed in bacteriophage λ are used to infect *Escherichia coli* cells carrying plasmids containing DNA sequences homologous to the desired gene. By the use of an appropriate combination of genetic markers, phage growth is made dependent on the occurrence of homologous recombination between the phage and plasmid genomes. Thus, only those bacterial cells that have received the desired sequence can give rise to plaques. Poustka *et al.* (8) have significantly expanded this approach by extending it to cosmid libraries. A disadvantage of these methods is that the newly isolated sequence could be disrupted by the recombination events, which complicates subsequent genetic and structural analysis of the cloned gene.

We describe here another way of conferring selectivity on a cloning vehicle. In this method, restriction fragments are generated whose sticky ends are characteristic of the locus rather than of the enzyme. Fragments of this type are readily produced by using enzymes that cut in a staggered manner at nonspecific sequences either within or flanking a specific binding site (9). The ability of such site-specific sticky ends to rehybridize efficiently with their complements has been shown by the reconstruction of plasmid and bacteriophage genomes by the perfect reassortment of up to seven *Bgl* I or *Hga* I restriction fragments (9, 10). This selectivity also could be used for the specific hybridization of rare restriction fragments in complex mixtures (11). The cloning of such specific fragments requires vectors that contain the very same restriction sites as those bounding the fragment. These sites should be available if the gene in question had been cloned previously by conventional means. After cutting both the vector and the genomic DNA with the same restriction enzyme, the vector should hybridize to the desired fragment and to a relatively small number of others that happen to have the same ends.

We have applied this principle for the efficient cloning of restriction fragments bearing the *dhfr* gene from CHO cells. Approximately a 500-fold enrichment of the *dhfr* gene can be achieved in cosmid libraries prepared from cells that carry

Abbreviations: DHFR, dihydrofolate reductase; *dhfr*, locus for DHFR; kb, kilobase(s); SV40, simian virus 40; *cos* site, cohesive-end site.

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only a single copy of this gene. This method can be of general use for the repetitive cloning of the same sequence using plasmid and phage as well as cosmid vectors.

MATERIALS AND METHODS

Materials. The enzymes used in this work were *E. coli* DNA polymerase Klenow fragment, T4 DNA ligase, and various restriction endonucleases from Bethesda Research Laboratories; T4 DNA polymerase I and various other restriction enzymes from New England Biolabs; and DNA polymerase I and calf intestinal alkaline phosphatase from Boehringer Mannheim. α-^{32}P-labeled deoxynucleotides were obtained from Amersham. Nitrocellulose filter sheets (BA85) were from Schleicher & Schuell. All other biochemicals were purchased from Sigma.

Elimination of the *Bgl* I Sites from the Cosmid Vector pGCcos3neo. The cosmid vector pGCcos3neo (provided by G. Crouse) carries the neomycin resistance gene (*neo*) of Tn*5*, linked to the simian virus 40 (SV40) early region transcription and processing signals, and the β-lactamase gene originally derived from pBR327 (12). *E. coli* transformed with this vector are resistant to neomycin or kanamycin (L. Graf, personal communication; unpublished results) as well as to ampicillin. The vector contains two *Bgl* I sites: one located within the ampicillin-resistance marker and one located within the SV40 origin. Vector DNA was cut with *Bgl* I, made blunt ended with T4 DNA polymerase (13), and religated. After transformation of *E. coli* HB101, neomycin-resistant colonies were selected and screened for insensitivity to *Bgl* I. One isolate, pGCB2, was chosen for further use.

Construction of the 5′ and 3′ *dhfr*-Specific *Bgl* I Cosmid Vectors. Southern blot (14) analysis of *Bgl* I-cleaved CHO genomic DNA in 0.4% agarose gels revealed a 41-kb fragment containing the intact *dhfr* gene. DNA containing the 5′ and 3′ *Bgl* I sites was obtained from two *dhfr* cosmids (H-2 and 1-1, respectively) isolated by Milbrandt *et al.* (15). From H-2, a 6.7-kb *Eco*RI fragment containing the *Bgl* I site nearest the 5′ end of the *dhfr* gene was first cloned into the *Eco*RI site of pBR325 (16). From 1-1, a 1.4-kb *Bam*HI–*Kpn* I fragment carrying the *Bgl* I site nearest the 3′ end was cloned into pSV2gpt (17). *Bgl* I-containing fragments were further subcloned from these two plasmids into the derivatized vector pGCB2. The piece selected from the 5′ end was a 2.0-kb *Hin*cII fragment and from the 3′ end was a 0.72-kb *Bam*HI–*Rsa* I fragment. These particular pieces were chosen to minimize sequences upstream of the 5′ site and downstream of the 3′ site in order to keep the aggregate size of the eventual *dhfr* cosmid to <49 kb. The overall scheme is depicted in Fig. 1. Each fragment was cloned into separate vector molecules. It was necessary to place each *Bgl* I-containing fragment on the same side of the *cos* sequence and in the same orientation so as to insure the packaging of essential plasmid genes. The *Bam*HI end of the 0.72-kb *Bam*HI–Rsa I fragment was ligated to the *Bam*HI-cut vector. After filling in the remaining *Bam*HI end with Klenow polymerase, we ligated the resulting blunt ends. The 2.0-kb *Hin*cII fragment was cloned into the *Eco*RI site, which had similarly been made blunt-ended. The ligation products were used directly to transform *E. coli* HB101. Neomycin-resistant colonies were screened for plasmids that could be cleaved with *Bgl* I. Two vectors with their inserts in the same orientation were chosen and designated pGCD5 (7.3 kb) and pGCD3 (6.0 kb). They are shown in Fig. 1.

Cosmid Cloning of *dhfr* Genes. Single-copy *dhfr* genes were cloned by using DNA that had first been size-fractionated. CHO DNA (300–500 μg) was cut with *Bgl* I and divided among 3 or 4 tubes containing 13.5 ml of a 4–12% sucrose gradient in TEN buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA/0.2 M NaCl). Centrifugation was at 20°C

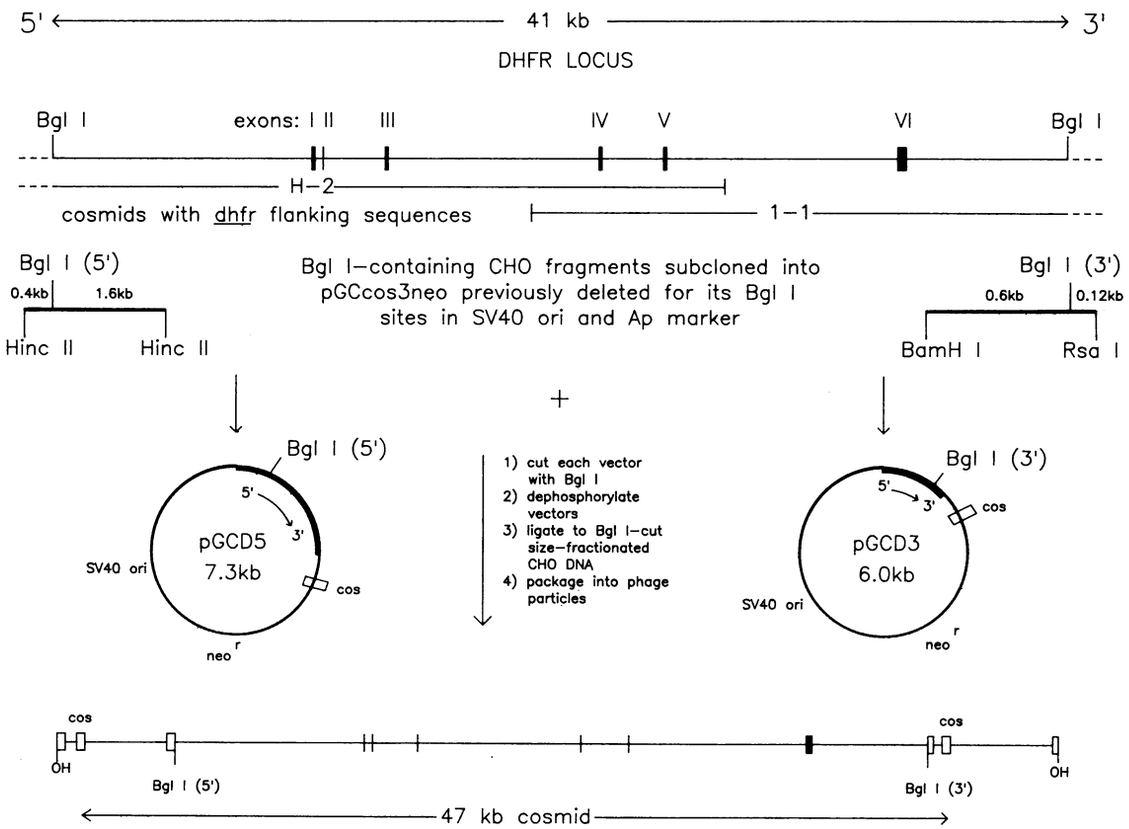

FIG. 1. Construction of custom cosmid vectors for cloning the Chinese hamster *dhfr* gene.

for 17 hr in a Beckman SW 40 rotor at 22,000 rpm. DNA of molecular size ≥35 kb is pelleted by this procedure. After removal of all but ≈0.4 ml of supernatant, an equal volume of TEN buffer was added, and the DNA was solubilized and precipitated with ethanol. The DNA was resolubilized in 0.5 ml of TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA), extracted three times with phenol and three times with ether, precipitated twice with ethanol, and solubilized in 0.1× TE at a concentration of 1 mg/ml. This DNA was ligated to the two *Bgl* I-cut, phosphatase-treated (13) vectors by using T4 ligase at 0.4 Weiss units/ml. The final concentrations were 500 μg/ml for genomic CHO DNA and 20 μg/ml for each vector. The reactions were terminated by adding EDTA (pH 8.0) to a final concentration of 10 mM and heating at 68°C for 15 min. The ligated DNA was then precipitated with ethanol and thoroughly resolubilized at 1 mg/ml in 0.1× TE for use in the packaging step.

The extent of the ligation could be monitored by Southern blot analysis (described in Fig. 2). Packaging was carried out as described (ref. 13, method II) with putrescine omitted. One packaging reaction was used for each 4 μg of CHO DNA. Cosmid vector DNA was found to inhibit the packaging reaction strongly: the maximum amount of combined vectors per packaging reaction was 350 ng. DH1 (18) was the recipient strain for cosmid infection. Overnight cultures were pelleted, resuspended in the original volume of 10 mM $MgSO_4$, and incubated at 37°C for 30 min. A sample of this suspension (1.2 ml) was mixed with the 0.5 ml of particles from each packaging reaction, incubated at 37°C for 20 min, and then diluted 1:2 with Luria broth (LB). After a 60-min expression period, the cultures were selected on LB agar plates containing 20 μg either of neomycin or of kanamycin per ml. Two-to-four plates were used per packaging reaction. Colonies were picked and placed in duplicate grid patterns on a nitrocellulose sheet on the surface of a 150-mm agar dish as well as an ordinary dish and grown overnight. Detection of colonies carrying *dhfr* sequences was carried out on the nitrocellulose sheet by the procedure of Hanahan and Meselson (19). The hybridization probe contained a mixture of cloned fragments representing 13 kb of sequence from the *dhfr* gene (5).

The recombinant cosmids carrying the *dhfr* gene were usually found to grow more slowly than other recombinants, as evidenced both in liquid culture and by their small colony size. Reconstruction experiments using one such slow-growing clone showed that plating efficiency was also variable. Maximum colony yield was obtained by inoculating cultures in top agar (containing 5 μg of kanamycin per ml) rather than by the usual spreading technique; therefore, this procedure was used routinely in the preparation of cosmid libraries.

The procedure for cloning the *dhfr* gene from the amplified mutant MK42 (20) was done in a similar way except that the size fractionation step was omitted.

Gene Transfer. The wild-type *dhfr* gene cloned from MK42 or UA21 (5) was assayed for biological activity by transfer into DHFR-deficient CHO mutants (5). Gene transfer was accomplished by the calcium phosphate coprecipitation technique (21) or by protoplast fusion (22). In the case of the former, 1 μg of plasmid DNA and 19 μg of carrier calf thymus DNA were used per 100-mm dish containing 2×10^6 recipient cells. Protoplast fusion was carried out essentially as described by Milbrandt *et al.* (23). In both procedures, cultures were allowed a 24- to 48-hr expression period, after which *dhfr*-positive transformants were selected by plating 2×10^6 cells per 150-mm dish in F-12 medium lacking hypoxanthine and supplemented with 10% (vol/vol) dialyzed fetal calf serum. Transformants arose at a frequency of 10^{-5} to 10^{-4}; control experiments in which no *dhfr* gene was present yielded no viable colonies among $>10^7$ cells tested.

General Procedures. Ligations were carried out at 4°C or 14°C for 18 hr (13). Conditions for restriction digestion, gel electrophoresis, and DNA fragment purification have been described (6).

RESULTS

Cloning Strategy. The *dhfr* gene in Chinese hamster cells consists of six exons spread over ≈26 kb of DNA (6, 15). With the goal of cloning the *dhfr* gene intact, we screened several restriction enzymes in a search for one that would not cut within the gene. *Bgl* I proved to be such an enzyme, with one site ≈8 kb upstream and one site 7 kb downstream from the 5′ and 3′ end of the gene, respectively (Fig. 1). Digestion of CHO cell DNA with *Bgl* I followed by electrophoresis in a 0.2% agarose gel and Southern blotting revealed the expected 41-kb fragment (data not shown). This size is an appropriate one for cloning in cosmid vectors (1, 2). More importantly, although *Bgl* I is a type II restriction enzyme, it cuts within a nonspecific sequence that interrupts the recognition site: the sequence G-C-C-N-N-N-N-N-G-G-C is cut after the seventh nucleotide as written (24–27). The resulting three-base 3′ overhang is derived from the nonspecific region in the center of the recognition sequence. Therefore, most *Bgl* I-generated restriction fragments have different ends. The chance that a fragment will have two particular ends is $1/4^3 \times 1/4^3 \times 2 = 4.9 \times 10^{-4}$. Cosmid vectors that include the same 5′ and 3′ *Bgl* I sites that flank the *dhfr* gene should be highly selective for the fragment containing that gene. This selectivity will be compromised by the occurrence of other fragments that are of a size able to be packaged in bacteriophage λ particles and that happen to have the same overhanging trinucleotide sequence at each end. The frequency of such non-*dhfr* fragments can be calculated as being only about 12 per cell, assuming 5 pg of DNA per CHO cell and ≈20% being of packageable size (30–45 kb) after *Bgl* I digestion. Of course, this predicted maximum frequency of 1 in 12 for the *dhfr* gene fragment would be expected to be further reduced by the extent of mismatching that is allowed during the hybridization and ligation procedure.

Construction of the Specialized Cosmid Vectors. Two cosmid vectors specialized for the cloning of *Bgl* I fragments containing the *dhfr* gene were constructed starting with the cosmid pGCcos3neo (12). This cosmid carries a neomycin-resistance gene under the control of the SV40 early promoter. This gene serves as a selectable dominant marker that can be expressed in animal cells as resistance to the antibiotic G418 (28). It also can be selected for in *E. coli* as neomycin or kanamycin resistance, since the bacterial promoter is still functional (L. Graf, personal communication; our data not shown). This last property is important because the first step in the modification of this vector was to remove its two *Bgl* I sites, one of which lies in the ampicillin-resistance gene. The ampicillin-resistance gene was inactivated by this procedure, leaving the neomycin-resistance gene as the only remaining drug-resistance marker (Fig. 1).

The regions from the *dhfr* locus that contain either the 5′ or 3′ *Bgl* I sites were then cloned into this modified cosmid (pGCB2). Two previously isolated cosmid clones (15) that span either the 5′ or the 3′ end of the *dhfr* gene were used as the source of these sequences. As illustrated in Fig. 1, a 2.0-kb *Hin*cII fragment containing the 5′ site was cloned into the *Eco*RI site of pGCB2. The 3′ sequence was inserted in the same orientation by cloning a 0.72-kb *Bam*HI–*Rsa* I fragment into the *Bam*HI site. When these two vectors (pGCD5 and pGCD3, respectively) are cut with *Bgl* I and ligated to the *Bgl* I fragment bearing the *dhfr* gene, a 53-kb molecule should result that has phage λ *cos* packaging sites spaced 47 kb apart. *In vitro* packaging of these molecules

should yield cosmids containing 6 kb of plasmid sequence and 41 kb of inserted mammalian DNA (Fig. 1).

Ligation of the *dhfr* Gene. As a preliminary test of the ability of these vectors to anneal specifically to the *dhfr* fragment in complex mixtures of DNA, we ligated them to a *Bgl* I digest of DNA from the CHO mutant line MtxRIII. These cells contain about 20 copies of the *dhfr* locus because of gene amplification (29), thus making the expected ligation products somewhat easier to detect. After ligation, the DNA was cut with *Eco*RI or *Hin*dIII plus *Kpn* I and was subjected to Southern blot analysis. The majority of the *dhfr* sequences at both the 5′ and 3′ ends of the 41-kb fragment became ligated to the corresponding vector under these conditions (Fig. 2). Similar results were obtained with cell line UA21 (5), which contains only a single copy of the *dhfr* gene.

Cloning the *dhfr* Genes. For our first cloning experiment, we utilized the methotrexate-resistant clone MK42, since this mutant contains 300 copies of the *dhfr* gene as a result of gene amplification (20). At this high frequency, virtually all of the cosmid colonies in a *Bgl* I library should represent the *dhfr* gene. If the ligation of the *Bgl* I ends were truly selective, a low overall frequency of neomycin-resistant cosmid colonies would be expected. This prediction was borne out: an efficiency of only 21 colonies per μg of MK42 DNA was achieved. That the packaging extracts were highly active was indicated by control experiments in which ≈10^8 plaque-forming units of bacteriophage λ were produced per μg of phage λ DNA. Twenty-one cosmids from the MK42 ligation were examined for the presence of restriction fragments characteristic of the *dhfr* locus. Seven displayed the expected *Kpn* I, *Bam*HI, and *Bst*EII fragments. One of these cosmid clones (pDM16) was then tested for the presence of a functional *dhfr* gene by protoplast fusion (21, 23) with a CHO *dhfr* deletion mutant. Transformant colonies with a DHFR$^+$ phenotype (purine independence) were produced at a frequency of 2×10^{-4}. Control protoplasts yield no

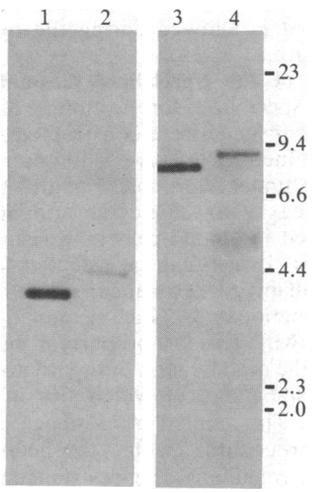

FIG. 2. Ligation of the *dhfr Bgl* I fragment in complex mixtures to the 5′ and 3′ cosmid vectors. Total unfractionated DNA from clone MtxRIII was cleaved with *Bgl* I and then ligated to a mixture of *Bgl* I-cleaved cosmids pGCD5 and pGCD3. A portion of the ligation product was then cut with *Eco*RI and subjected to Southern blotting analysis with a hybridization probe from the 5′ end of the 41-kb *Bgl* I *dhfr* fragment (a 1.5-kb *Sma* I–*Eco*RI fragment located 6 kb upstream of exon I). The results are shown in lanes 1 (unligated MtxRIII DNA) and 2 (ligated). Another portion of the ligation product was cut with *Hin*dIII and *Kpn* I and was subjected to Southern blot analysis with a probe from the 3′ end (a 0.7-kb *Bam*HI fragment 0.8 kb downstream of exon VI). These results are shown in lanes 3 (unligated) and 4 (ligated). The size markers are in kilobases and represent radioactively labeled phage λ *Hin*dIII fragments.

Table 1. Summary of *dhfr* cloning experiments

DNA	Amount, μg	No. of colonies screened	*dhfr*$^+$ colonies found
MK42	1	21	7
UA21	20	118	1
UA21	26	335	2
DS11	24	363	2
DS31	18	331	1
DU92	28	576	2
Total single-copy DNAs: (all except MK42)		1723	8 (0.46%)

Clones DS11, DS31, and DU92 represent DHFR-deficient mutants that will be described elsewhere.

DHFR$^+$ colonies among 10^7 cells. The activity of pDM16 was confirmed by using the DNA–calcium phosphate coprecipitation technique (21). Restriction digests of the remaining 14 cosmids did not yield the electrophoretic pattern expected for the *dhfr* locus.

We next focused on CHO clone UA21, a hemizygote that contains only a single copy of the *dhfr* locus (5). Since UA21 DNA contains only 1/300th of the *dhfr* genes in a preparation from MK42, it was necessary to use a much greater amount of DNA. In preliminary experiments, the high concentrations of genomic and vector DNA necessary to effect ligation were found to inhibit the packaging reaction. Therefore, a size fractionation of the *Bgl* I-cut genomic DNA was added to the protocol. Restriction fragments ≳35 kb were pelleted in a sucrose gradient; ≈80% of the DNA was removed by discarding the supernatant solution. Several hundred cosmid colonies could be generated from 20 μg of the pelleted DNA after ligation and packaging. These colonies were transferred to nitrocellulose sheets, grown further, amplified, and then screened for the presence of *dhfr*-related sequences by the method of Grunstein and Hogness (30) as described in *Materials and Methods*. Positive colonies were easily discernible in the rectangular grid (data not shown). Once again, positive colonies yielded the predicted restriction pattern for the *dhfr* locus and were able to confer a DHFR$^+$ phenotype by gene transfer to *dhfr*-deficient cells.

The cloning of the *dhfr Bgl* I fragment from single-copy DNA was repeated four additional times, including three cases in which *dhfr*-deficient mutants were the sources of DNA. The results of these five experiments and the initial result with the amplified mutant are summarized in Table 1. The overall frequency of *dhfr*$^+$ cosmids is approximately 1 in 200. Whereas this number falls short of the theoretical maximum of 1 in 12, it still represents an enrichment of several hundred-fold over that expected from the cloning of random DNA fragments. Most importantly, by using this procedure, the *dhfr* gene can be reliably isolated by screening only 200–300 colonies on a single nitrocellulose filter.

DISCUSSION

The use of cosmid vectors that have been custom designed for cloning the Chinese hamster *dhfr* gene has enabled us to isolate this single-copy gene routinely by the hybridization screening of only 200–300 bacterial colonies. Two factors have contributed to the enrichment of the *dhfr* gene. We were fortunate in that this gene is contained in a restriction fragment of ≈41 kb, making it an ideal size for cloning in a cosmid. In theory, the size constraints of the phage λ packaging system should have been able to provide the selectivity necessary to effect a size fractionation for molecules in this range. However, because of the inhibitory effects of high DNA concentrations in the packaging re-

action, we found it more expedient to reduce the total amount of DNA that was to be ligated. Size fractionation of the restriction digest results in about a 5-fold purification of this fragment. Since all of these fragments are of packageable size when ligated to the vectors, no further enrichment is expected on the basis of packaging selectivity. The major factor in the enrichment is due to the use of restriction fragments with heterogeneous single-stranded ends. These ends are most conveniently produced by restriction enzymes that cut in a nonspecific sequence that is different from their recognition sequence. However, they also may be produced by alternative means such as exonucleases. Within the size-fractionated DNA, the *dhfr* gene is represented once in about 25,000 molecules (assuming an average fragment size of 40 kb and a total genome size of 5×10^6 kb). Thus, the contribution of the custom cloning vehicle to the enrichment is a factor of $\approx$100. This number is also roughly consistent with the result from the one experiment using DNA from the amplified mutant MK42, where the frequency of positive colonies using unfractionated DNA was 1 in 3.

The final frequency of positive colonies (1 in 200) fell somewhat short of that predicted on theoretical grounds (1 in 12). There are several possible reasons that could account for this. The most obvious and most probable is that the occasional ligation of mismatched ends takes place in the face of the overwhelming number of such ends in the ligation reaction. Another possibility is that the assumption of a random distribution of sequences is unwarranted; that is, the particular sequences found at the *Bgl* I sites flanking the *dhfr* gene may be ones that are repeated many times in the genome. Whatever the reason, the important result is that the final number of recombinant colonies that need to be screened by the Grunstein–Hogness procedure is small enough to fit on a single filter.

The strategy used here could be used whenever there is a need to repetitively isolate the same stretch of DNA from many clones or individuals of a given species. Some examples include the cloning of polymorphic or mutant genes from a series of individuals, the cloning of mutant oncogenes from independent tumors, and, as described here, the cloning of mutant genes from cultured cell lines. In the present work, DNA was cloned into cosmid vehicles because of the large size of the *dhfr* gene and our desire to clone the entire gene in a single fragment. However, the incorporation of cloned restriction sites characteristic of a locus could just as well be carried out with plasmids or double- or single-stranded phage vectors. In addition to *Bgl* I, there are several other commercially available restriction enzymes, both type I and type II, that are able to yield the heterogeneous staggered ends required by this method. Without the constraint of isolating a locus in one piece, it should be possible to clone any region (in two or more pieces) by making use of one or more of these available restriction enzymes. Moreover, the method need not be used in its most selective form in order to be of value. The utilization of only one site (i.e., only one end of the fragment to be cloned) for hybridization selectively should provide an enrichment of about a factor of 10 (the square root of the factor of 100 seen here). This single order of magnitude can represent a significant savings in labor and expense when screening many large libraries. Recently, Manos and Gluzman (31) have used this one-selective-end approach as the final step in cloning integrated SV40 sequences from transformed cells. Such a strategy also may be useful for cloning partial deletion mutations (e.g., ref. 5) or for cloning rearrangements where one end of the locus remains constant—e.g., immunoglobulin or T-cell receptor genes.

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