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Cosmid vectors for rapid genomic walking, restriction mapping, and gene transfer

(cloning vectors/genomic DNA libraries/gene mapping/chromosome walking)

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ABSTRACT **We have designed cosmid vectors for rapid genomic "walking" and restriction mapping. These vectors contain the transcription promoters from either bacteriophage SP6, T7, or T3 flanking a unique *Bam*HI cloning site. Mammalian expression modules encoding the dominant marker neomycin phosphotransferase or the amplifiable dihydrofolate reductase gene expressed from SV40 promoters were inserted for use in gene transfer studies. Restriction sites for the enzymes *Not* I and *Sfi* I, which cut mammalian DNA very infrequently, have been engineered near the transcriptional promoters to enable the excision of most inserts as single, full-length fragments. Genomic libraries representative of mouse, human, and hamster genomes were constructed by inserting 33- to 44-kilobase-pair (kbp) DNA fragments, generated by partial cleavage of genomic DNA with *Mbo* I or *Sau*3A, into the unique *Bam*HI site. Digestion of recombinant cosmids with restriction enzymes that cleave frequently but do not disrupt the transcriptional promoters generates two small DNA templates for the synthesis of end-specific RNA probes to facilitate directional "walking." Cosmid restriction maps can be determined rapidly by one of several methods. The cosmids and methods we describe should have wide utility in determining the functional and structural organization of complex eukaryotic genomes and for physically linking distant genetic loci.**

The ability to clone and characterize large segments of eukaryotic DNA is important for the structural and functional analysis of mammalian genomes and the isolation of defective genes responsible for human disease. The use of convenient molecular fingerprints generated by restriction site polymorphisms, chromosome breakpoints, or other chromosome anomalies to regionally identify the loci associated with Huntington disease (1), cystic fibrosis (2, 3), and retinoblastoma (4) should enable the isolation and characterization of the affected genes. However, with the present methodology, a formidable amount of effort is required to traverse the distance from sites of close linkage to the genes in question. In the absence of convenient chromosomal deletions or inversions to move a restriction marker site closer to the target gene, more laborious procedures for finding the gene of interest such as isolating sequentially overlapping genomic clones ("walking") (5) or directional cloning of fragments separated by several hundred kilobase pairs (kbp; "jumping") (6) are required.

"Walking" from one site to another along a chromosome is generally achieved by using bacteriophage vectors capable of propagating 20–25 kbp inserts, or cosmid vectors that can propagate more than 45 kbp of contiguous genomic DNA. Cosmids are plasmids that contain the bacteriophage λ *cos* (cohesive-end site) sequences enabling the *in vitro* packaging of recombinant molecules with a minimum size of 38 kbp and a maximum size of 52 kbp (78% and 105% of phage λ, respectively). Due to their larger cloning capacity, cosmids have proven to be valuable cloning vehicles for the isolation of sequentially overlapping clones to define much of the mouse major histocompatibility complex (7, 8), to map extensive regions around the mouse T locus (9), and to elucidate the structure of amplified DNA in drug-resistant mammalian cells (10).

Early cosmid vectors contained only plasmid origins of replication, bacterial genes specifying antibiotic resistance, and the bacteriophage λ cohesive termini (11–14). More recently, specialized cosmid vectors and accompanying methods have been developed to enable transfection and selection in mammalian cells (15, 16) and *Drosophila* (17), to allow rescue of transfected sequences from mammalian cells (18), to facilitate homologous recombination between cosmids (19), or to aid restriction endonuclease mapping using *cos* cohesive ends (20). However, none of these vectors is specifically designed to enable the efficient isolation of sequentially overlapping cosmid clones in a genomic "walking" procedure or for the functional mapping of cloned DNA fragments. We report the construction of a series of specialized cosmid cloning vectors that, in addition to replication and selection functions present in earlier vectors, contain bacteriophage transcriptional promoters placed to allow rapid and efficient restriction mapping and genomic "walking."

MATERIALS AND METHODS

Cosmids pCV107 and pCV108 (16) were provided by Y. W. Kan, pGEM2 and SP6 polymerase were from Promega Biotec (Madison, WI), and T3 and T7 polymerases and *Eco*K-deficient bacteriophage λ *in vitro* packaging lysate (Gigapack and Gigapack Gold) were supplied by Stratagene Cloning Systems (San Diego, CA). [α-^{32}P]dCTP and [α-^{32}P]UTP were from New England Nuclear and ICN.

Construction of Cosmid Vectors. The pWE cosmid vectors containing SP6, T7, and T3 promoters are shown in Fig. 1. Further details of the construction of these cosmid vectors will be presented elsewhere (21).

Construction of Genomic Libraries. Genomic libraries were prepared according to the methods of Dillela and Woo (22) using both standard and *Eco*K-deficient (Gigapack Gold, Stratagene Cloning Systems) packaging extracts. The cloning efficiencies of this insert DNA (modal size = 40–60 kbp) ranged from 2×10^4 to 5×10^5 colonies per μg of insert DNA, and library sizes ranged from 4×10^5 to 6×10^6 independent cosmid clones.

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Abbreviations: kbp, kilobase pairs; CHO, Chinese hamster ovary; *dhfr*, locus for dihydrofolate reductase (7,8-dihydrofolate:NADP$^+$ oxidoreductase; EC 1.5.1.3) in CHO cells.

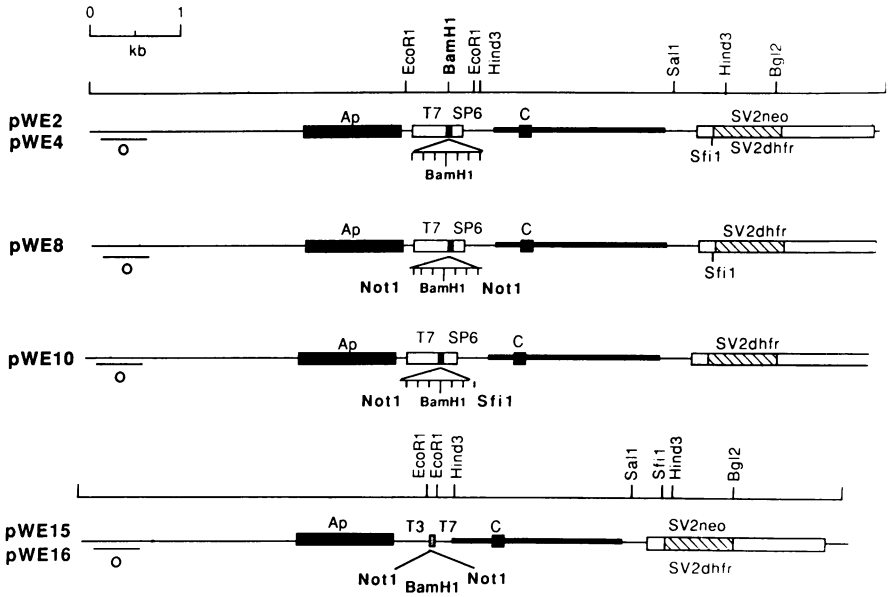

FIG. 1. The structure of pWE cosmid vectors. The construction of the pWE vectors is described in *Materials and Methods* and ref. 21. pWE2 and pWE4 contain the polylinker region of plasmid pGEM2. Introduction of coadhesive synthetic oligonucleotides into the *Bam*HI site of pWE4 has added the additional restriction sites for *Not* I and *Sfi* I in pWE8 and pWE10. SV2neo and SV2dhfr represent neomycin/kanamycin (G-418) phosphotransferase and dihydrofolate reductase selectable genes expressed from SV40 promoters (16). O, plasmid origin of replication; Ap, β-lactamase gene; and C, bacteriophage λ *cos* site. Relevant restriction sites present in the polylinker sequences as well as the location of the bacteriophage T7, T3, and SP6 promoters are shown. An *Sfi* I site present in the SV40 sequence was removed by site-specific mutation in the pWE10 vector. The structures of cosmid vectors pWE15 and pWE16 are shown in the last diagram. The locations of bacteriophage promoters and relevant restriction sites in the cloning region of each cosmid vector are also shown. pWE15 and pWE16 were constructed by inserting synthetic oligonucleotides with *Eco*RI-coadhesive termini into pWE2 or pWE4 digested with *Eco*RI.

Rapid Restriction Mapping. The parental pWE vector and all of the pWE recombinants studied give high yields of DNA (>5 μg of cosmid per ml of bacterial culture) when grown in small-scale culture, and we have not observed deletion, rearrangement, or recombination when the cosmids are propagated in *Escherichia coli* strain DH5 (Stratagene Cloning Systems) or its derivatives. One can perform restriction mapping or walking without resorting to large-scale cultures. Cosmid DNA (5–20 μg, purified by either CsCl density-gradient centrifugation of large-scale bacterial preparations or by phenol extraction of rapid lysates) was partially digested, phenol extracted, and the DNA was collected by ethanol precipitation. One-microgram samples were transcribed with the SP6, T7, or T3 polymerase for 1 hr at 37–40°C. Transcription reactions were done according to Melton *et al.* (23) using 50 μCi (1 Ci = 37 GBq) of [α-^{32}P]UTP and 500 μM unlabeled UTP to insure the synthesis of transcripts longer than 10 kb. The reactions were terminated by phenol extraction and ethanol precipitation in the presence of 50–100 μg of carrier yeast RNA, and the precipitates were resuspended in 50 μl of sterile H_2O. The [α-^{32}P]UTP incorporation was determined by trichloroacetic acid precipitation. Approximately 10^6–10^7 cpm of freshly prepared probe were size-fractionated on 1% formaldehyde/agarose gels as described previously (24, 25). End-labeled, *Hin*dIII-digested λ DNA run on the same gels provided size standards. Following electrophoresis, the gels were dried and autoradiographed for 1–30 min at room temperature. A simpler mapping procedure for pWE15,16, which does not involve transcription, is described in *Results*.

Preparation of Riboprobes for Chromosome Walking. Recombinant cosmid DNA was digested to completion with a four-nucleotide-specific restriction endonuclease that does not cleave within the SP6, T7, or T3 promoters (e.g., *Hae* III or *Rsa* I), purified by phenol extraction, and collected by ethanol precipitation; 1–2 μg of DNA was transcribed in a 20-μl reaction as described by Melton *et al.* (23) using 50 μCi of [α-^{32}P]UTP and 12 μM unlabeled UTP. Transcription reactions were phenol extracted, transcripts were collected by ethanol precipitation in the presence of 50–100 μg of carrier yeast RNA, and the riboprobes were used for hybridization without removal of the DNA template. Hybridization reactions were done in 0.25 M $NaHPO_4$, pH 7.2/0.25 M NaCl/1% $NaDodSO_4$/1 mM EDTA/50% formamide/100 μg of Ficoll per ml/100 μg of polyvinylpyrrolidone per ml/100 μg of bovine serum albumin per ml/200 μg of denatured salmon sperm DNA per ml/200 μg of yeast tRNA per ml. Colony filters were hybridized at 42°C for 10 min, 1–5 × 10^6 cpm per ml of ^{32}P-labeled end-specific RNA probe was added, and hybridization was carried out for 12 hr at 42°C. Filters were washed in 0.1× SSC (SCC: 0.15 M NaCl, 0.015 M sodium citrate, 1 mM EDTA)/0.1% $NaDodSO_4$ at 50–65°C for 1–2 hr.

Gene Transfer. DNA-mediated gene transfer by calcium phosphate precipitation was done as previously described (26). Transfection by electroporation was performed using a BTX T-100 transfector (Biotechnologies and Experimental Research, San Diego, CA) and previously published methods (26).

RESULTS AND DISCUSSION

Design and Properties of pWE Cosmid Vectors. These studies were motivated by the need for a high-capacity cosmid vector that would facilitate the analysis of the functional and structural organization of mammalian genomes. Such a vector should ideally contain several features in addition to the required bacterial replication origin, drug resistance gene, and bacteriophage *cos* sequences. First, the design should allow for rapid production of probes specific for both ends of the inserted sequence to facilitate bidirectional chromosome walks away from the cloned DNA. Second, the vector should facilitate restriction mapping of the insert to expedite the generation of a structural map of large

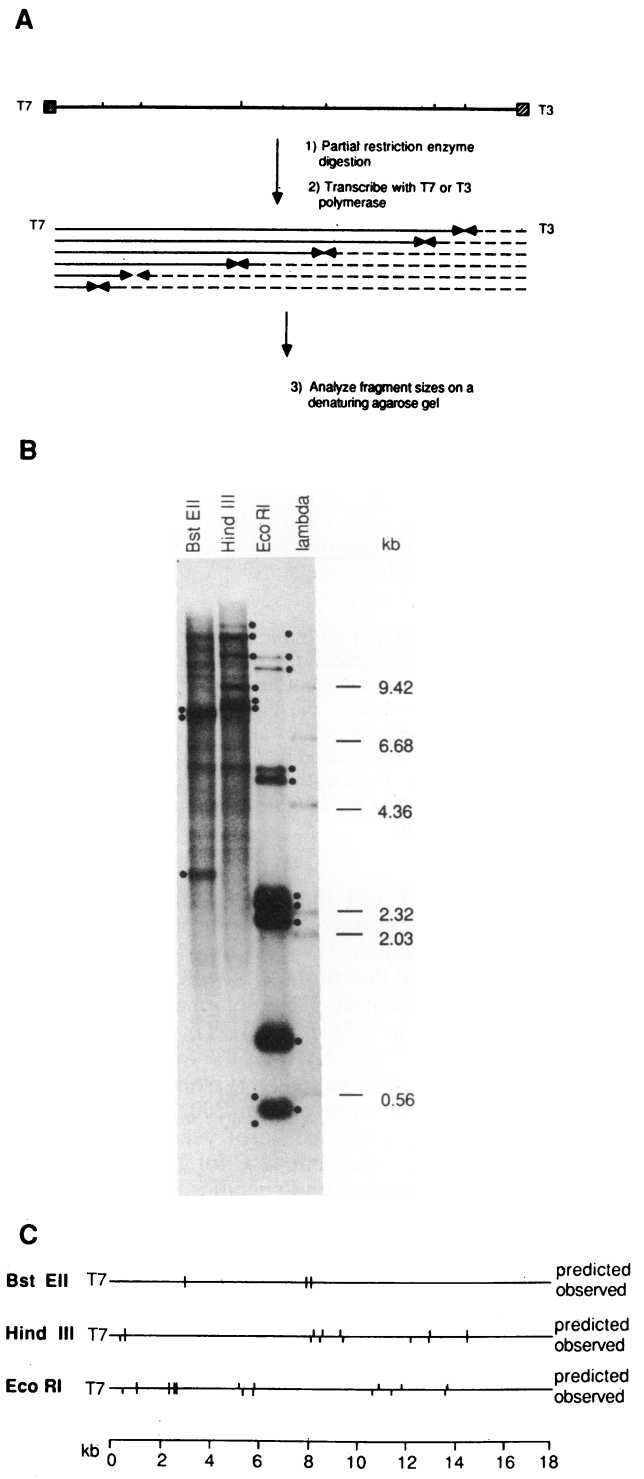

FIG. 2. (*A*) Strategy for restriction mapping using pWE cosmids. (*B*) RNA synthesis from the T7 promoter of partially digested cosmid DNA. A pWE2 cosmid clone from a CHO library containing a 1000× amplification of the *dhfr* gene was digested with *Eco*RI, *Bst*EII or *Hin*dIII to give a distribution of partial digestion products and RNA synthesis done with T7 polymerase. Radiolabeled RNA transcripts (≈10^6 cpm per lane) were analyzed on a 1% formaldehyde/agarose gel (25). Radiolabeled HindIII fragments of bacteriophage λ were included as size markers. The gel was dried and exposed to XR5 film for 5 min at room temperature. (*C*) Derivation of a restriction map from RNA transcripts. The maps shown were derived from the data in *B*. The locations of restriction sites determined from published data (29, 30) (predicted) are compared to those determined by RNA synthesis (observed). The positions of all transcripts used to generate the maps are indicated by the dots (the largest *Eco*RI transcript and two smallest *Hin*dIII transcripts are obvious upon longer exposures). The transcription products that are identical for all three enzymes were excluded from consideration because it is unlikely that they result from termination at restriction termini. Once this background of prematurely terminated and/or degraded transcripts is subtracted, the sites depicted remain.

chromosomal regions and to detect regions of overlap between different recombinant cosmids. The recombinant molecules should also be propagated at a sufficiently high copy number to allow probes to be prepared directly from DNA isolated from rapid lysates (27), thereby obviating the requirement for time-consuming large-scale cosmid DNA preparations. Third, it is often advantageous to be able to determine whether a cloned DNA contains a functional gene. Inclusion of a dominant selectable or amplifiable marker gene would enable one to first select for transformants containing the donated cosmid inserted into a functional chromosomal region, or to increase the copy number and expression of the cloned sequences, prior to assaying for their function. Finally, in some cases it would be useful to be able to remove the entire 40–45 kbp insert as a single restriction fragment [as for injection into embryos to establish transgenic animals (28)].

The pWE cosmid vectors illustrated in Fig. 1 fulfill all of the requirements enumerated above. The prototype cosmid vectors pWE2 and pWE4 are derived from cosmids pCV107 and pCV108 (16) by inserting a restriction fragment containing bacteriophage T7 and SP6 promoters flanking a synthetic polylinker. Recognition sequences for the rare restriction enzymes *Not* I and *Sfi* I were added using synthetic oligonucleotides, creating pWE8 and pWE10. These vectors are useful for the construction of genomic libraries and restriction mapping, but "walking" has been difficult for the following reason. All vectors constructed with the pGEM2 transcription module contain a minimum of 30 transcribed nucleotides before the *Bam*HI insertion site. This "linker-leader" sequence is common to all transcripts made from such vectors and contributes to significant nonspecific background hybridization when screening cosmid libraries with end-specific probes (see below). In pWE15,16, the first nucleotide added by either polymerase is only four nucleotides from the insertion site, a distance too short to enable stable hybrids to be formed under the hybridization and washing conditions routinely used. To allow convenient excision of inserts lacking *Not* I sites, these restriction sites were included upstream of each bacteriophage promoter.

Restriction Mapping by Transcription from pWE Recombinants. Bacteriophage promoters located near the ends of the insert DNA provide a convenient and rapid means of restriction endonuclease mapping. One strategy for restriction mapping pWE cosmids is shown in Fig. 2*A*. Recombinant cosmid DNA is partially digested with any restriction enzyme to generate a collection of fragments that should provide a representation of the cleavage sites present. The fragment mixture is then transcribed *in vitro* under conditions that allow for the synthesis of transcripts larger than 10 kb (see *Materials and Methods*). Each transcript produced should indicate the distance between the site of cleavage and the position of the first transcribed nucleotide. Fractionation of these transcripts according to size reveals the position of each cleavage site relative to the promoter. Because the two promoters in each vector are positioned to allow for transcription into the insert, the same set of fragments produced by partial digestion allows one to restriction-map from both ends of the inserted sequence by merely setting up two transcription reactions.

Fig. 2*B* shows an example of the transcript patterns generated from cosmids containing a portion of an amplified Chinese hamster ovary (CHO) *dhfr* (dihydrofolate reductase)

gene. Transcription of partial *Eco*RI digests with T7 polymerase reproducibly generates discrete RNA species that range in size from approximately 0.5 kb to >13 kb (the latter transcript is indicated by a dot in Fig. 2*B* and can easily be seen on longer exposures). The restriction map defined by these fragments (Fig. 2*C*) agrees with previously published restriction maps (31, 32). Partial *Bst*EII- or *Hin*dIII-digested templates give several large transcription products as well as a higher background. Since *Eco*RI sites are more numerous and more closely spaced than the *Bst*EII and *Hin*dIII sites, we infer that the high background is due to premature termination or degradation (or both) of the long transcripts produced in the latter reactions. Nevertheless, the *Bst*EII and *Hin*dIII restriction maps defined by these transcripts (there are two small *Hin*dIII transcripts at the positions indicated by the dots that appear on longer exposures) are also in good agreement with previously published data (31, 32) with the exception of one additional *Hin*dIII site detected in these experiments (possibly a restriction site polymorphism). It has been possible to map 10–15 kb from each promoter using the procedure described, and longer distances could potentially be mapped by employing different gel concentrations and longer reaction times. The method is limited only by the extent of premature termination and RNA degradation and the ability to resolve and size large RNA molecules on denaturing gels. However, all methods that depend upon gel analysis for determination of restriction maps (29, 30) are limited to the same degree by the resolution of the gel systems employed.

An alternative restriction mapping strategy that avoids transcription-related problems and is more rapid and convenient than the method described above has been developed for use with vectors such as pWE15,16. Recombinant cosmids are digested partially as described above, but they are then digested to completion with *Not* I to generate a set of fragments that begin at the T7 or T3 promoters and end at the site of cleavage of the first enzyme (unless there is an intervening *Not* I site). These *Not* I-terminated partial digestion products are then fractionated on an agarose gel and blotted to a solid support. The fragments can then be mapped relative to the T7 or T3 promoters by hybridizing the blot with end-labeled oligonucleotide-sequencing primers specific for these promoters (available commercially).

Chromosome Walking Using Riboprobes Generated by Transcription of pWE Cosmids. pWE vectors were designed to place bacteriophage promoters at each end of a cloned genomic DNA insert so that RNA probes synthesized from these promoters would generate end-specific hybridization probes. To minimize the probability of a repetitive DNA sequence being present in the end-specific probe, cosmid DNA is digested with a restriction endonuclease (*Hae* III or *Rsa* I) that cuts mammalian DNA frequently but does not disrupt either promoter. The short, promoter-proximal transcripts generated by transcription of cosmid DNA digested with such enzymes is then hybridized with colony filters to detect overlapping clones. As a test of this procedure, cosmid clones were isolated from a CHO cell genomic library containing a 1000-fold amplification of the *dhfr* gene with a *dhfr* cDNA probe, and an end-specific "walking" probe synthesized from a pWE15dhfr cosmid clone. Hybridization to duplicate library filters (Fig. 3*A*) demonstrated the presence of three classes of hybridizing clones: (*i*) those hybridizing only to a *dhfr* cDNA probe, (*ii*) those hybridizing to both cDNA and end-specific "walking" probes, and (*iii*) those hybridizing to the "walking" probe alone. The last class contains "steps" in the genomic "walking" procedure away from the *dhfr* reference clone, as might those in the second

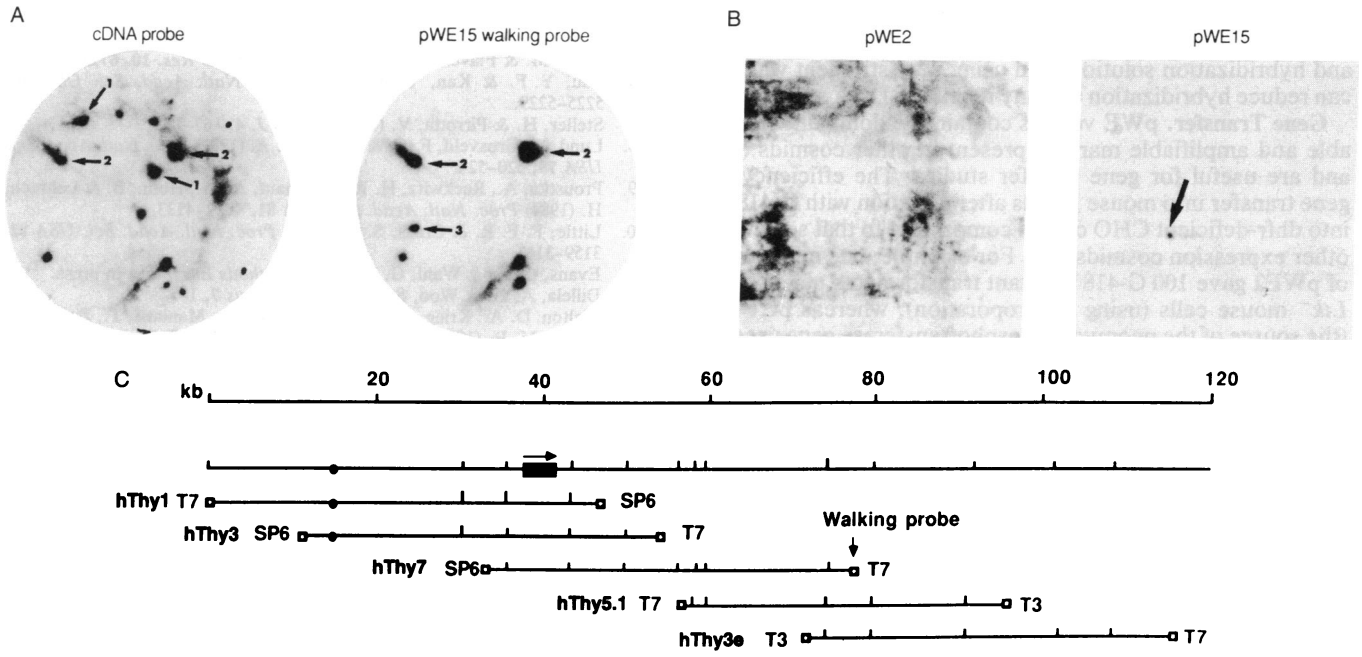

FIG. 3. (*A*) Genomic walking from an amplified *dhfr* gene. A *dhfr* cDNA probe and an end-specific T7 transcript derived from one pWE15dhfr cosmid were hybridized to duplicate filters of a pWE15 genomic cosmid library constructed using CHO DNA containing a 1000× amplification of the *dhfr* gene. Hybridizing colonies annotated as 1 indicate those hybridizing to the cDNA probe alone; 2 indicates those colonies hybridizing to both cDNA and T7 walking probe; 3 indicates a clone hybridizing to the "walking" probe alone and represents a "step." (*B*) Genomic walking with unique sequence genes. A "walking" probe was synthesized from the T7 promoter of a pWE2 cosmid clone with a 42-kb insert containing the human *Thy-1* gene. It was then hybridized to both a pWE2 and a pWE15 cosmid library constructed using human placenta DNA. A clone representing a "step" in the walk is identified by an arrow. Note the significant difference in nonspecific hybridization of the *same probe* to libraries constructed in a cosmid with the pGEM transcription casette (pWE2) or the modified transcription casette in pWE15. (*C*) *Eco*RI restriction map of 120 kbp of DNA surrounding the human *Thy-1* gene on chromosome 11q23. A T7 "walking" probe synthesized from cosmid hThy7 was used to isolate overlapping clones. The small closed circles represent the site of a probable restriction fragment length polymorphism when this map is compared to a previously determined map of a portion of this region (33). The location of the *Thy-1* gene (bar) and direction of transcription are indicated. Cosmid clones isolated using the pWE "walking" procedure are shown in boldface.

class. One clone that hybridized only with the end-specific probe was purified, and Southern blotting analysis confirmed the region of overlap. An end-specific probe prepared from this clone showed that it was part of the amplified unit when it was hybridized to total genomic DNA isolated from the wild-type and highly amplified CHO cells.

To further confirm the utility of this procedure for walking through unique sequence regions, several cosmid clones containing the gene for the human neural antigen Thy-1 were isolated from a pWE2 human genomic library using a mouse cDNA probe (34). A T7 end-specific probe was synthesized from one cosmid clone and used to screen human genomic libraries prepared in both pWE2 and pWE15. Fig. 3*B* shows the presence of a clone hybridizing to an end-probe in the pWE15 library. The pWE2-derived probe shows no background when hybridized to the pWE15 library, but this probe shows significant background when hybridized to the pWE2 library: this background is due to hybridization of the "linker-leader" sequence present in each transcript and in each cosmid containing the pGEM2 riboprobe module. Purification of several hybridizing clones and determination of their restriction maps revealed the regions of overlap. These clones define a 120-kbp region of human DNA surrounding the Thy-1-encoding locus (data not shown). The restriction map of the overlapping pWE clones agrees with a previously determined map, with the exception of a single *Eco*RI site, which may be attributed to a restriction fragment length polymorphism (33).

The presence of repetitive DNA sequences within end-specific probes compromises their use because such probes would hybridize to many clones in the library. However, there are several strategies for overcoming problems caused by such repeats: (*i*) a different restriction enzyme can be used to prepare template DNA, (*ii*) a different overlapping cosmid clone for "walking" probes can be used, or (*iii*) adding 10–20 μg of denatured genomic DNA per ml to the prehybridization and hybridization solutions and using very stringent washes can reduce hybridization of many repetitive DNA sequences.

Gene Transfer. pWE vectors contain the dominant selectable and amplifiable markers present in other cosmids (16) and are useful for gene transfer studies. The efficiency of gene transfer into mouse L cells after selection with G-418 or into dhfr-deficient CHO cells is comparable to that seen with other expression cosmids (16). For example, one microgram of pWE2 gave 100 G-418 resistant transformants per 5×10^5 Ltk^- mouse cells (using electroporation), whereas pCV108 (the source of the neomycin phosphotransferase gene used in pWE2) gave 60 transformants per 5×10^5 mouse Ltk^- cells (16) (using the calcium phosphate coprecipitation method). Transfection using electroporation has generally allowed gene transfer at much higher efficiency into mouse myeloma cells and other lymphoid cells than calcium phosphate-mediated gene transfer (data not shown). pWE cosmids containing different genomic DNA inserts give efficiencies of 3–25 transformants per 5×10^5 cells per μg of DNA (either $dhfr^+$ transformants of CHO $dhfr^-$ cells, or G-418 resistant transformants of mouse cells).

Conclusions. The data presented here demonstrate the utility of using pWE cosmids for obtaining representative genomic libraries, for isolating unique genes, and for rapid restriction mapping and chromosome walking. The use of efficient "walking" and restriction mapping, coupled with efficient gene transfer into eukaryotic cells, should facilitate the physical mapping and functional analysis of mammalian genomes.

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Note Added in Proof. The correct orientation of the T7 and SP6 promoters in pWE2 is the opposite of that shown in Fig 1.

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