Chromosome-specific recombinant DNA libraries from the fungus *Aspergillus nidulans*

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ABSTRACT

Development of physical genomic maps is facilitated by identification of overlapping recombinant DNA clones containing long chromosomal DNA inserts. To simplify the analysis required to determine which clones in a genomic library overlap one another, we partitioned *Aspergillus nidulans* cosmid libraries into chromosome-specific subcollections. The eight *A. nidulans* chromosomes were resolved by pulsed field gel electrophoresis and hybridized to filter replicas of cosmid libraries. The subcollections obtained appeared to be representative of the chromosomes based on the correspondence between subcollection size and chromosome length. A sufficient number of clones was obtained in each chromosome-specific subcollection to predict the overlap and assembly of individual clones into a limited number of contiguous regions. This approach should be applicable to many organisms whose genomes can be resolved by pulsed field gel electrophoresis.

INTRODUCTION

Detailed genetic and physical maps of organisms' genomes provide an extremely useful starting point for cloning genes that are important to human health, agriculture, and industry (1–4). In many instances the only practical approach toward physical isolation of a gene involves 'walking' from an identified, previously cloned chromosomal site across the sought after locus (5,6). A genetic map consists of a set of genes whose linear order is established by recombinational linkage, whereas a physical map consists of an ordered set of specific sequences, for example restriction sites, often identified in cloned, overlapping DNA segments. These overlapping segments can correspond to large contiguous chromosomal regions termed 'contigs.' Contig maps have been completed for *Escherichia coli* (7,8) and are nearly complete for the nematode *Caenorhabditis elegans* (9). Efforts are being made to complete similar maps in other organisms including *Saccharomyces cerevisiae* (yeast) and human. The existence of contig maps facilitates gene cloning and makes possible novel investigations of the relationship of genome structure to function.

In vitro reconstruction of genomes into contigs is made much easier if recombinant DNA libraries are divided into chromosome-specific subcollections. Detection of overlap between clones within these restricted subcollections could generate contigs more readily than with unfractionated collections by reducing the number of clones subjected to analysis. One approach to the formation of such libraries is to initiate library construction from chromosomes that have been partially purified by flow cytometry (10,11) or by pulsed field gel electrophoresis (PFGE) (12–15). In many instances, however, it would be useful to sort pre-existing random libraries that have been extensively characterized and have well known properties into chromosome-specific subcollections. This is the case for two intensively investigated filamentous fungi, *Aspergillus nidulans* and *Neurospora crassa*. Many well characterized genes have been cloned from these organisms and located in existing cosmid collections. Similar collections are being made for industrially important species such as *Cepahalosporium acremonium*, the producer of the antibiotic cephalosporin (16,17), and plant pathogens such as *Magnaporthe grisea*, the causal agent of one of the most important diseases of plants, rice blast (18,19,20,).

Fungi possess small genomes and have chromosomes that can be separated by PFGE (21–25). Most fungi contain low amounts of repetitive DNA, almost all of which consists of rDNA occurring as a long tandemly repeated array of elements. The remainder of the reiterated DNA consists mainly of short, low copy, interspersed repeats. Based on these observations we reasoned that it should be possible to order existing genomic DNA libraries according to chromosome by using PFGE-isolated chromosomes as colony filter hybridization probes.

A. nidulans was chosen to test this idea for four reasons. First, two complete cosmid libraries in LORIST2 (26) and pWE15 (27) vectors have been constructed. LORIST2 and pWE15 possess bacteriophage Lambda and ColE1 origins of replication, respectively. DNA sequences poorly maintained in one vector are often stable in the other (27). Use of two libraries may therefore reduce problems of non-random representation. Additionally, numerous cloned genes have been identified within these collections. Second, the *A. nidulans* genome has been extensively characterized by classical genetic analysis, leading to the assignment of over 400 loci to its eight linkage groups (28). Third, the *A. nidulans* genome has been thoroughly

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investigated at the molecular level. The genome is small ($2.6-3.1\times10^7$bp) (23,25), and contains an exceedingly low amount of repetitive DNA as estimated by solution reassociation studies (23) and by Southern blot analyses of genomic DNA with cloned chromosomal fragments (unpublished results). Finally, application of contour-clamped homogeneous electric field (CHEF) (15) electrophoresis of DNA from wild type and reciprocal translocation strains permits separation of the eight *A. nidulans* chromosomes, each of which has been assigned to a genetic linkage group (25).

MATERIALS AND METHODS

Preparation of Chromosomal DNA

Aspergillus nidulans protoplasts were prepared as described (25) from strains FGSC 4 (wild type), and reciprocal translocation strains FGSC 40 [T1(V;VI)], and 2499 [T1(I;III)] (provided by E. Käfer). The protoplasts were washed twice with STC buffer (1.2 M sorbitol/10mM Tris-HCl, pH 7.5/10 mM CaCl2). They were then pelleted by centrifugation at 4000 rpm in a Sorvall HB-4 rotor for 5 min at 4°C and resuspended in GMB buffer (0.125 M Na-EDTA, pH 7.5/0.9 M sorbitol) at a concentration $3-4\times10^8$/ml. The suspension was placed at 42°C, and an equal volume of molten 1.4% InCert agarose (FMC) in GMB buffer cooled to 42°C was added. The protoplasts were gently resuspended and poured into a plug mold and solidified on ice for 10 minutes. Plugs were immersed in 50°C NDS buffer (0.5 M Na-EDTA, pH 8.0/10 mM Tris-HCL, pH 9.5/1% sodium N-lauroylsarcosinate) containing proteinase K (1 mg/ml) for 18 hours. Plugs were then washed three times for 30 min in 50 mM Na-EDTA (pH 8.0) and stored in the same buffer at 4°C.

Gel Electrophoresis

Pulsed field gel electrophoresis was performed with a CHEF-DRII system (BioRad Laboratories). A 100 ml gel containing 0.8% Megarose (Clontec) was poured directly into a mold in the apparatus. The DNA-agarose plugs were inserted into the gel well and sealed with 0.8% Seaplaque agarose (FMC) in 0.5× TAE buffer (29). Gels were electrophoresed at 12°C in 0.5× TAE buffer at 48 V with three pulse intervals of 50 min (73 hr), 45 min (18 hr), and 37 (73 hr). Gels were stained in ethidium bromide (0.5 μg/ml) for 45 min and then destained in water for 1 hr.

Preparation and Radiolabeling of Chromosome-Specific DNA

Gel-resolved chromosome was cut out of the gel and DNA was extracted by glass bead adsorption (Bio-101). Chromosome-specific DNA (100−200 ng DNA/band) was labeled to specific activities > 1×10^8 dpm/μg using the random hexamer priming method (30), with [α-^{32}P]dCTP (3000 Ci/mmole; 1 Ci = 37 GBq) (Amersham).

Transfer and Hybridization

CHEF gels were blotted to membranes as described (25). Hybridizations were carried out for 18 hr at 68°C in 0.5 M NaCl/0.1 M Na-phosphate, pH 7.0/6 mM Na-EDTA, pH 8.0/1% SDS/denatured salmon sperm DNA (100 μg/ml) at a probe concentration of $1-2\times10^6$ dpm/ml. Blots were then washed at 68°C twice for 20 min in 2× SSC/1% SDS (1× SSC = 0.15 M NaCl/0.015 M Na-citrate, pH 7.0) twice for 20 min in 0.5× SSC, and subjected to autoradiography.

Two random *Aspergillus* cosmid libraries were transferred to Hybond-N membrane (Amersham). Cell lysis and prehybridization were performed as recommended by the manufacturer. Chromosome-specific hybridization probes were used at concentrations of $1-4\times10^5$ dpm/ml. Hybridization and washing were carried out as for Southern blots. The filters were subjected to autoradiography at −70°C for 1−5 days with intensifying screen and Kodak XAR film.

RESULTS AND DISCUSSION

The extent of cross hybridization between individual *A. nidulans* chromosomes was assayed to determine the applicability of the proposed approach. Gel-isolated chromosomes were radiolabeled and used to probe Southern transfers of CHEF-resolved chromosomes of wild type and reciprocal translocation strains. The products of the reciprocal translocation between chromosomes V and VI in strain FGSC 40 resolves chromosomes I and III. The products of the strain 2499 translocation between chromosomes I and III resolves chromosomes V and VI. Figure 1 shows that, with two exceptions, each chromosome hybridized predominantly with itself, confirming that the gel-resolved chromosomes could be isolated with a high degree of purity and that repeated DNA sequences were not a major problem. The exceptions involved chromosomes II, V, and VI: chromosomes II and VI when used as probes gave strong positive hybridization signals with chromosome V (Figure 1, lanes 5, 6, 10, 11). By contrast, chromosome V when used as probe hybridized primarily with itself (Figure 1, lane 9). This nonreciprocity of hybridization, which was repeatable, may be due to unequal representation of homologous repetitive DNA sequences on different chromosomes. If the radiolabeled probe is in sequence excess, these results suggest that a repeated element present in many copies on chromosome V is present in only a few copies

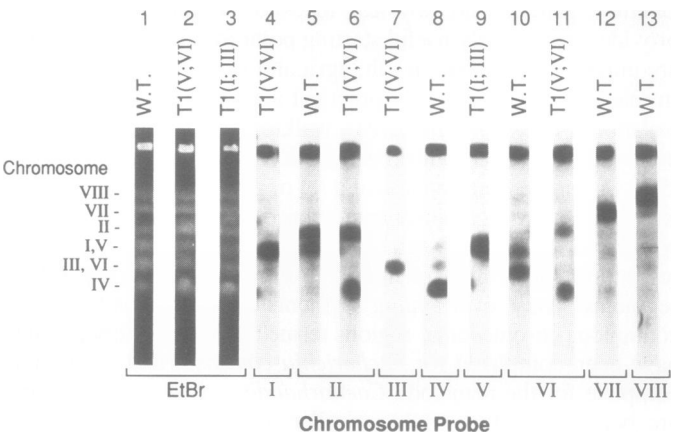

Figure 1. Specificity of gel-isolated *A. nidulans* chromosomes for use as hybridization probes. Chromosomal DNA from *A. nidulans* strains FGSC 4 (wild type), and two strains with reciprocal translocations, FGSC 40 [T1(V;VI)] and 2499 [T1(I;III)] was resolved by CHEF electrophoresis. Lanes 1−3: ethidium bromide stains of the wild type (lane 1) and reciprocal translocation strains. Lanes 4−13: Resolved chromosomes were transferred to nylon membranes and hybridized with radiolabeled gel isolated chromosomes.

on chromosomes II and VI. Interestingly, the rDNA tandem repeat, which mapped to chromosome V by CHEF gel blot hybridization with a cloned rDNA probe (31, 32; unpublished results) also weakly hybridized with chromosomes II and VI (data not shown), indicating that a few copies of a sequence related to the rDNA repeat were represented on these chromosomes as well. The unequal distribution of the rDNA-complementary sequences on chromosomes II, V and VI could account for the observed pattern of chromosome cross hybridization.

Chromosome cross hybridization raised the question of whether repeated DNA sequences might interfere with assignment of clones to linkage groups. However, short, diverged, interspersed repetitive elements should not be a problem, because hybridization at high stringency would result in a weak signal due to the small fraction of the insert of any clone that would be stably complementary to the probe. Similarly, long tandem repeats might not be a problem because only a limited number of clones are expected to cross-hybridize.

Figure 2 shows a representative hybridization experiment in which a radiolabeled chromosome probe was hybridized with filter replicas of a cosmid library. Strong hybridization signals were observed with ~10% of the 960 clones, the expected genomic proportion of chromosome V (Table 1), whereas the remainder produced weak or negligible signals. These results indicated that CHEF-resolved chromosomes could be radiolabeled to specific activities sufficient to permit unambiguous identification of complementary clones.

Eight copies of the *A. nidulans* genomic libraries (5134 clones/copy) were subjected to colony hybridization with radiolabeled, gel-isolated chromosomes. The hybridization data were analyzed by using a contig mapping and analysis program package, called CMAP, with a VAX 6210 computer . A majority of the cosmids (64%) hybridized uniquely with isolated chromosomes. In a parallel experiment, the probes were hybridized with replicas of *S. cerevisiae* colonies containing artificial chromosomes (YACs) with ~150 kb *A. nidulans* DNA inserts (H. B., W. E. T. and John Carbon, unpublished results).

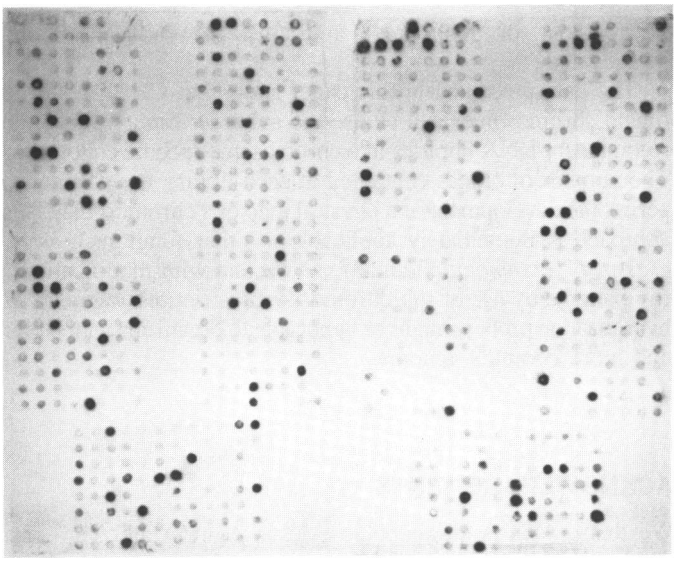

Figure 2. Colony hybridization analysis with *A. nidulans* chromosome V as probe. A random sample of 960 *A. nidulans* cosmid clones was hybridized to CHEF-resolved chromosome V.

Unique hybridization patterns were obtained, although the signals were weaker than with cosmid clones (data not shown).

To confirm the chromosome-specificity of the subcollections, cosmids hybridizing with varying degrees of intensity to single chromosomes were picked into chromosome-specific subcollections. Copies of single microtiter dishes from each subcollection were again hybridized with radiolabeled, gel isolated chromosome probes. Figure 3 shows that most selected clones produced strong hybridization signals with the complementary probes and gave weak or negligible signals with heterologous chromosomes. A fraction of cosmids originally assigned to a single chromosome failed to hybridize with their proposed chromosome of origin but did hybridize strongly with one or more other chromosomes. In all cases, these unexpected signals were traced to erroneous inclusion of clones in the subcollections. Another small fraction of clones hybridized weakly with the proposed chromosome of origin and with one or more other chromosomes. A possible explanation for these ambiguous clones was the incorrect inclusion of weakly hybridizing clones as positives. In addition, we have observed that some clones maintained through several transfers on plates lose significant fractions of inserted DNA. Loss of cloned DNA could account for decreased hybridization intensity in these screens.

Eight chromosome-specific subcollections consisting of a total of 3307 clones were obtained by this method. Table 1 summarizes the number of clones per chromosome and the lengths of chromosomes in terms of physical (mbp) and recombinational (mu) size. Regression analysis of the number of identified chromosome-specific clones versus chromosome size showed a correlation ($r=0.78$) (33), supporting the hypothesis that all chromosomal regions have an equal probability of being cloned and selected. The probabilities of each chromosome-specific collection containing a specific region were >0.95, assuming random representation (33). However, exclusion from the subcollections of clones that hybridized with more than one chromosome may result in nonrandom gaps in contigs.

Of the 5134 cosmids examined, 1033 hybridized with two chromosomes and 736 hybridized to two or more chromosomes so that the clones could not be assigned unambiguously to a single chromosome. The observation that this hybridization pattern was repeatable indicated that it was not due to experimental noise.

Table 1. Chromosomal coverage based on chromosome-specific subcollections.

Chromosome	Size (mb)	Size (mu)	(kbp)/(mu)[a]	Cosmids in collection	Probability of coverage
I	3.8	280	13.6	461	0.98
II	4.2	340	12.3	410	0.98
III	3.5	270	13.0	432	0.99
IV	2.9	118	24.6	343	0.98
V	3.8	185	20.5	326	0.96
VI	3.5	274	12.8	276	0.95
VII	4.5	410	11.0	468	0.98
VIII	5.0	390	12.8	591	0.99
	Σ 31.2	Σ 2267	x15.0	Σ 3307	x 0.98

Chromosome lengths (mbp) are taken from reference 25. Sizes in map units (mu) were calculated from the genetic map of *A. nidulans* (28) taking regions >50 mu to be 50. The cosmids in each subcollection are only those that hybridized uniquely with chromosomal probes. To determine the correspondence between physical and genetic distances, chromosome physical lengths were divided by genetic lengths. The expected proportion of coverage was calculated by using the approach of Fu *et al.* (33).

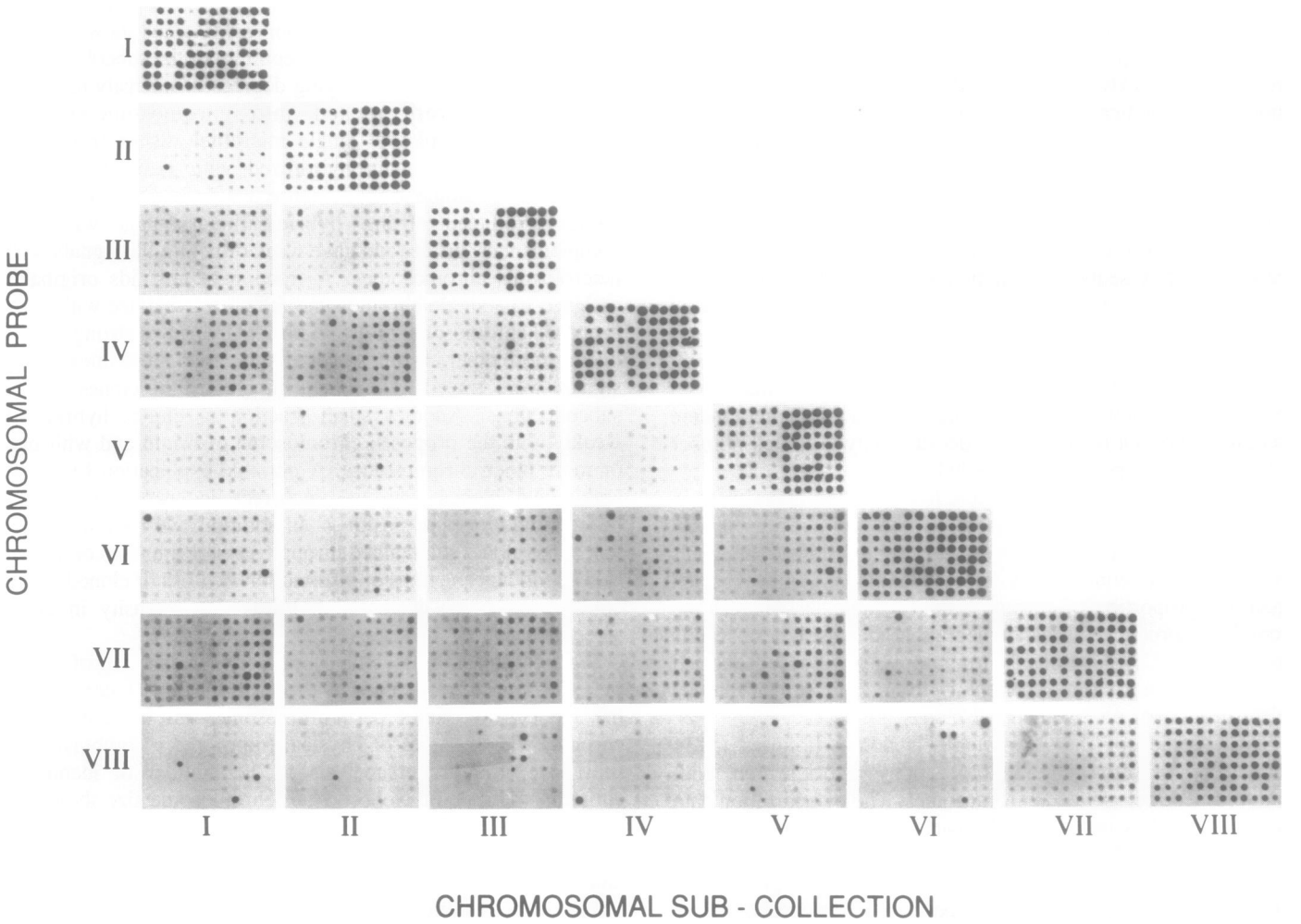

Figure 3. Hybridization specificity of chromosome-specific subcollections. Cosmid clones hybridizing with a single chromosome probe were picked to microtiter dishes to form chromosome-specific subcollections. The left half of each microtiter dish contained DNA cloned into the cosmid LORIST2, the right half contained *A. nidulans* DNA cloned into the cosmid pWE15. Chromosome-specific DNA probes were used to probe replica-filters of each subcollection.

In order to produce a strong hybridization signal, clones must have significant sequence identity across their entire length with the hybridization probe. Thus, most clones containing typical interspersed repetitive DNA sequence elements of ~ 500 bp are not expected to hybridize extensively with multiple chromosomes. Conserved, repeated sequence elements of >3–4 kb, however, could cause significant cross hybridization. Interestingly, the number of clones hybridizing with more than one chromosome had little correlation with the extent of chromosomal cross homology. Chromosomes II and VI, which cross hybridized strongly with chromosome V (Figure 1), did not share homology with more clones than did the other chromosomes, supporting the notion that these two chromosomes have a low number of copies of a repeated element present in multiple copies on chromosome V, probably rDNA. Cross hybridizing clones can now be examined for the presence and properties of repeated sequences. Assignment of clones that hybridize with two (or more) chromosomes to unique classes will assist in formation of contig sets from additional data.

A third class of 58 clones was identified that hybridized strongly with all eight chromosome probes. It is possible that these clones contain long, conserved sequence elements associated with centromeres or telomeres, and this possibility is being investigated.

The data presented in this paper show that CHEF-resolved fungal chromosomes can be used to suborder random genomic recombinant DNA libraries into chromosome-specific collections. The number of clones contained in each library is sufficient to permit the development of a physical map of a chromosome. This approach is immediately applicable to other fungi such as *N. crassa*, *C. acremonium*, and *M. grisea* and with modifications, for example by use of sub-chromosome restriction fragments as hybridization probes, might be applicable to organisms with larger and more complex genomes.

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