Physical Map of a Conditionally Dispensable Chromosome in *Nectria haematococca* Mating Population VI and Location of Chromosome Breakpoints

Jürg Enkerli,*1 Heather Reed,1 Angela Briley,1 Garima Bhatt,2 and Sarah F. Covert1

*Department of Botany and 1Daniel B. Warnell School of Forest Resources, University of Georgia, Athens, Georgia 30602

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**ABSTRACT**

Certain isolates of the plant pathogenic fungus *Nectria haematococca* mating population (MP) VI contain a 1.6-Mb conditionally dispensable (CD) chromosome carrying the phytoalexin detoxification genes MAK1 and PDA6-1. This chromosome is structurally unstable during sexual reproduction. As a first step in our analysis of the mechanisms underlying this chromosomal instability, hybridization between overlapping cosmid clones was used to construct a map of the MAK1 PDA6-1 chromosome. The map consists of 33 probes that are linked by 199 cosmid clones. The polymerase chain reaction and Southern analysis of *N. haematococca* MP VI DNA digested with infrequently cutting restriction enzymes were used to close gaps and order the hybridization-derived contigs. Hybridization to a probe extended from telomeric repeats was used to anchor the ends of the map to the actual chromosome ends. The resulting map is estimated to cover 95% of the MAK1 PDA6-1 chromosome and is composed of two ordered contigs. Thirty-eight percent of the clones in the minimal map are known to contain repeated DNA sequences. Three dispersed repeats were cloned during map construction; each is present in five to seven copies on the chromosome. The cosmid clones representing the map were probed with deleted forms of the CD chromosome and the results were integrated into the map. This allowed the identification of chromosome breakpoints and deletions.

**C H R O M O S O M E - l e n g t h polymorphisms and chromosomal instability have been described in a variety of fungi (for review see Zolan 1995). Changes in chromosome size and number have been observed primarily during sexual reproduction (Miao et al. 1991; Plummer and Howlett 1993; Zolan et al. 1994; Leclair et al. 1996), but there are also examples in which changes occurred during vegetative growth (Ruschenko-Bulgac et al. 1990; Talbot et al. 1993; Horsten et al. 1996). The mechanisms underlying chromosome instability and karyotype changes in filamentous fungi have not been studied directly. It has been proposed, however, that repeated sequences dispersed in the genome are responsible for creating polymorphisms by serving as sites for recombination within or between chromosomes (Zolan 1995). Chromosome instability may be one mechanism involved in genome evolution that contributes to the adaptability of fungal organisms to environmental changes (Zolan 1995).

Certain isolates of the ascomycete fungus *Nectria haematococca* mating population (MP) VI contain a 1.6-Mb conditionally dispensable (CD) chromosome (Covert et al. 1998). This supernumerary chromosome is not essential for the normal, saprophytic growth of *N. haematococca* MP VI, but it does contribute to the disease-causing capacity of this pathogen on chickpea plants (Enkerli et al. 1998). As a consequence, its presence provides an adaptive advantage when *N. haematococca* MP VI is growing in chickpea-containing environments. Two functional genes (MAK1 and PDA6-1), both encoding enzymes that detoxify phytoalexins, have been cloned from this chromosome (hereafter referred to as the MAK1 chromosome; Reimann and VanEtten 1994; Covert et al. 1996).

The MAK1 chromosome is unstable during sexual reproduction (Miao et al. 1991). When two isolates containing the 1.6-Mb chromosome were crossed, a variety of genetic events affecting the 1.6-Mb chromosome were detected including loss, nondisjunction, and truncation (Miao et al. 1991). The fact that truncated chromosomes of the same size were present in ascospore isolates from different tetrads suggested that hotspots for chromosome breakage might be present on the MAK1 chromosome (Miao et al. 1991). After more than 10 years of laboratory subculturing, the MAK1 chromosome has never been lost or truncated during mitotic cell division, except when exposed to benomyl (S. F. Covert, unpublished results; VanEtten et al. 1998).

The MAK1 chromosome of *N. haematococca* MP VI is a good experimental system for the analysis of chromosome rearrangement and instability because it does not contain essential genes. As a result, structural changes...
in the chromosome are not lethal and it is possible to recover all types of chromosomal derivatives. In addition, because the instability of this chromosome is observed at a high frequency during a defined point in the life cycle (sexual reproduction), newly formed derivatives of the chromosome can be identified readily. We have begun to exploit the utility of this experimental system by constructing a physical map of the MAK1 chromosome. The map was built from a chromosome-specific cosmid library by simultaneously labeling both ends of cosmid clones and using them as probes to detect overlapping clones. During this process, three dispersed, repeated sequences on the chromosome were identified and mapped. The chromosome-specific library of the MAK1 chromosome was then probed with truncated derivatives of the chromosome that were generated during sexual reproduction. By integrating these hybridization results with the physical map, it was possible to identify deleted pieces of the chromosome, map breakpoints, and formulate hypotheses about the timing of different truncation events.

**MATERIALS AND METHODS**

_Fungal isolates:_ N. haematococca MP VI isolates used in this study are listed in Table 1. All isolates were grown and maintained on V8 agar medium (Miller 1955) at 26°C in the dark.

_DNA manipulations:_ Cosmid DNA was prepared using the Wizard miniprep DNA purification system (Promega, Madison, WI). Genomic DNA was isolated as described by Cover et al. (1996). Southern blot hybridizations (except map probing, see below) were carried out at 65°C according to the aqueous solution protocol described by Ausubel et al. (1987). Probes were synthesized using the DECAprime II DNA labeling kit (Ambion, Austin, TX). Cosmid end-specific probes for identification of terminal clones in contigs were synthesized with the same kit, except the random primers were exchanged with T3 or T7 primers (6.25 ng in a 12.5-μl labeling reaction) and the reaction was incubated for only 3 min. All blots were washed at 65°C twice with 2× SSC (1× = 0.15 m NaCl, 0.015 m Na₂-citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS), and once with 0.5× SSC, 0.1% SDS for 20-30 min.

All polymerase chain reactions (PCR) were performed using standard conditions according to supplier’s protocols (GIBCO BRL, Gaithersburg, MD). To close gaps in the map, Taq DNA polymerase was used for standard PCR amplification (94°C for 60 sec, 60°C for 60 sec, 72°C for 3 min, 30 cycles) and ELongase enzyme mix was used for long-distance PCR amplifications (denaturing for 3 min at 94°C; 35 cycles of 94°C for 30 sec, 68°C for 20 min). PCR fragments were cloned using the pGem-T vector system (Promega). The single-strand PCR reaction to synthesize the radiolabeled telomere-derived probe was performed with Taq DNA polymerase, 500 nm primer homologous to the telomeric repeat of Fusarium oxysporum (GGTTAGGTTAGG; Powell and Kistler 1990), and genomic DNA of isolate 156-30-6 as template (denaturing for 3 min at 94°C; 30 cycles of 94°C for 45 sec, 40°C for 60 sec, 72°C for 3 min). The deoxynucleotide concentrations in this reaction were 10 μM for dCTP, dGTP, and dTTP; 1 μM for dATP; and 0.33 μM for [α-32P]dATP (3000 Ci/mmol; NEN Life Science Products, Boston, MA).

DNA to be separated by contour-clamped homogeneous electric field (CHEF) electrophoresis was prepared as described in Enkerli et al. (1997). All samples were separated on a CHEF DR III unit (Bio-Rad, Hercules, CA). Conditions for separating the entire karyotype were as follows: 0.8% agarose in 0.5× TAE (40 mm Trisacetate, pH 8.5, 2 mm EDTA), 106° reorientation angle, 2 V/cm, 1200-2400 sec switch time, 72 hr, 14°C. Conditions for isolation of the MAK1 chromosome were as follows: 0.8% agarose in 0.5× TBE (1× = 89 mm Trisborate, pH 8.3, 2 mm EDTA), 106° reorientation angle, 3 V/cm, 500 sec switch time, 48 hr, 14°C. Conditions for separation of DNA cut with rare-cutting restriction enzymes (NotI or SwaI) were as follows: 0.8% agarose in 0.5× TBE, 120° reorientation angle, 6 V/cm, 10 sec switch time, 16 hr, 14°C. Chromosomal DNA bands were isolated from CHEF gels using the Elu-Quik DNA purification kit (Schleicher & Schuell, Keene, NH). Inagarose NotI or SwaI digestions were carried out as described by Birren and Lai (1993). Each plug was digested with 30 units of restriction enzyme for 20 hr.

Nucleotide sequencing was carried out on a PE sequencer (Applied Biosystems, Foster City, CA) at the Molecular Genetics Instrumentation Facility at the University of Georgia (Athens, GA). Sequences were analyzed with the Lasergene software package (DNASTar, Madison, WI).

### Table 1

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Size of MAK1 chromosome (Mb)</th>
<th>Mak phenotype</th>
<th>Pda phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>156-30-6</td>
<td>1.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>230-25-7</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>272-1-1</td>
<td>1.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>272-1-5</td>
<td>1.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>272-6-1</td>
<td>1.5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>272-6-2</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>272-26-1</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Isolates originally described by Miao et al. (1991) and Miao and VanEtten (1992). Their tripartite names indicate the cross, ascus, and ascospore numbers from which they were isolated, e.g., 156-30-6 is from cross 156, ascus 30, and ascospore 6. Isolates from cross 272 are progeny of 156-30-6 and 230-30-6, another isolate containing the 1.6-Mb MAK1 chromosome.

* Ability to detoxify maackiain (a chickpea phytoalexin), encoded by MAK1.

* Ability to detoxify pisatin (a pea phytoalexin), encoded by PDA6-1.

### Selection of a chromosome-specific library:

An existing MAK1 chromosome-specific library in cosmid pMOcosX (Orbach 1994) containing 280 clones (Covert et al. 1996) was supplemented with additional clones to increase its redundancy. Colony blots of 2304 new genomic clones from 156-30-6 were probed with the gel-isolated 1.6-Mb chromosome as described by Covert et al. (1996). Clones with a high-intensity hybridization signal (H clones) and clones with a moderate-intensity hybridization signal (M clones) were added to the chromosome-specific library and the probing was repeated to confirm chromosome specificity. The MAK1 chromosome does not hybridize to cloned ribosomal DNA (rDNA), but several cosmids containing rDNA were found in the chromosome-specific library (data not shown). During CHEF electrophoresis sheared fragments of the highly abundant rDNA apparently contaminated the chromosomal band used to select the chromosome-specific library. Therefore, all rDNA clones contaminating the chromosome-specific library
were identified by probing with pUF8-3, a rDNA fragment isolated from N. haematococca MP VI (Boehm et al. 1994). Hybridizing clones were removed from the chromosome-specific library and as a result the numbering scheme of the original chromosome-specific library (Cover et al. 1996) was changed. This caused clone 1G8 from which the MAK1 gene was cloned (Cover et al. 1996) to be renamed 1F7. The final chromosome-specific library consisted of 442 clones and was maintained in 5-microtiter plates (Sambo o k et al. 1989). The average insert size was estimated from 10 randomly chosen restriction-enzyme-digested cosmids. The complete chromosome-specific library had a redundancy of 9.7. Considering only the 209 H clones, the library had a redundancy of 4.6. At this level there was a 99% probability that each sequence on the chromosome was represented in the library (Seed et al. 1982).

**Map probing:** The complete chromosome-specific library was copied with a high-density replicator (V & P Scientific Inc., San Diego) onto Hybond-N nylon membranes according to the manufacturer’s protocol. Cosmid pMoCosX includes T3 and T7 priming sites that flank either side of the cosmid insert (Orbach 1994). Probes for mapping were radiolabeled by simultaneous T3/T7 primer extension reactions using the DECAprime II DNA labeling kit (Ambion) and [α-32P]dCTP. Random primers in the kit were replaced with a T3/T7 primer mix (3.1 ng each in a 12.5-μl labeling reaction) to label both ends of the cosmid insert. The incubation time for the labeling reactions was 15 min. DNA-DNA hybridizations with map probes were done in 6× SSC, 0.5% SDS at 65°C (Sambo ok et al. 1989). Blots were washed at 65°C twice with 2× SSC, 0.1% SDS and once with 0.5× SSC, 0.1% SDS for 20-30 min. Each hybridization reaction contained duplicate filters of the chromosome-specific library and only clones that hybridized to the probe on both filters were considered positive.

**Map assembly:** The random cost algorithm (Wang et al. 1994), as implemented in the fungal genome database (Kochut et al. 1993), was used to assemble the physical map of the MAK1 chromosome.

**RESULTS**

**Mapping strategy:** A MAK1-chromosome-specific cosmid library constructed in cosmid pMoCosX was used as the basis for this mapping project. The library consisted of 442 clones stored in 5-microtiter plates. Clones were divided into two groups on the basis of their hybridization intensity to the isolated MAK1 chromosome. “H clones” produced a highly intense signal in two separate hybridizations and were considered to be very likely from the 1.6-Mb MAK1 chromosome (total number of H clones = 209). “M clones” produced a moderately intense signal and were considered to potentially originate from another chromosome (total number of M clones = 233).

Probes to map the MAK1 chromosome of N. haematococca MP VI isolate 156-30-6 were selected on the basis of “sampling without replacement” (Hoheisel et al. 1993; Mizukami et al. 1993). During this process cosmids were selected as probes only if they had not served previously as probes or hybridized to a probe. This type of nonrandom probe selection is much more efficient than random probe selection for map construction (Palazzolo et al. 1991; Zhang and Marr 1993). The result of each probing was recorded in a binary code (positive or negative hybridization signal) for each clone in the chromosome-specific library. Thus, as sampling without replacement proceeded, each clone in the library developed a binary “fingerprint.” The binary hybridization matrix was analyzed by a computer program that assembles overlapping clones into contigs on the basis of the random cost algorithm (Wang et al. 1994). It is a way complementary ordering process in which the information gained by detecting overlapping clones is used to order the probes in a contig (Prade et al. 1997).

**Identifying clones containing repeated DNA sequences:** Cross-hybridization caused by repeated DNA sequences is a significant source of problems in physical map construction (Mayer et al. 1992; Cohen et al. 1993; Prade et al. 1997). Consequently, several steps were taken to identify clones that contained repeated DNA sequences so that their use as map probes could be avoided. As a first step, duplicate filters of the chromosome-specific library were probed with genomic DNA from two isolates, 230-25-7 and 272-26-1, which both lack the MAK1 chromosome. This procedure identified clones containing DNA sequences that are present on other chromosomes (e.g., centromeres, telomeres, and transposons). Thus, the 248 clones that hybridized to the genomic DNA from these isolates were termed R clones, for repeat clones. The 194 nonhybridizing clones were termed S clones, for single-chromosome probes or hybridized to a probe. This type of nonrandom probe selection for map construction (Palazzolo et al. 1991; Zhang and Marr 1993). The result of each probing was recorded in a binary code (positive or negative hybridization signal) for each clone in the chromosome-specific library. Thus, as sampling without replacement proceeded, each clone in the library developed a binary “fingerprint.” The binary hybridization matrix was analyzed by a computer program that assembles overlapping clones into contigs on the basis of the random cost algorithm (Wang et al. 1994). It is a way complementary mapping process in which the information gained by detecting overlapping clones is used to order the probes in a contig (Prade et al. 1997).

**Assessing the effect of a repeated sequence on a probe:** Cosmid 1F7 contains Nht1, a putative transposon (Enkerli et al. 1997; GenBank accession no. U78574). It was used as a probe against the chromosome-specific library to assess the effect of a repeated sequence on the resulting hybridization. The chromosome-specific library was hybridized to 1F7-derived probes synthesized via two different techniques. For the first probe, the insert of 1F7 was gel isolated and labeled with random primers. For the second probe, only the insert ends of 1F7 were labeled by primer extension from the T3 and T7 primer sites flanking the 1F7 insert. The results of both hybridization reactions are shown in Figure 1. Both strong and moderate hybridization signals were detected when the complete 1F7 insert was used as the probe, whereas only strong signals were detected
tion that did not hybridize to cosmids 1F7 or 2H6 were initially chosen as probes. Once all of these clones were either used as probes or hybridized to a probe, it became necessary to use some repeat-containing cosmids as probes to saturate the map. Sampling without replacement was carried out until all H clones either were used as probes or hybridized to a probe. M clones were not used as probes because of the possibility that they originated from another chromosome.

Despite efforts to identify and avoid clones containing repeated sequences, some probes gave a hybridization pattern like that shown in Figure 1A. In these cases, only strongly hybridizing clones were recorded as positive signals in the binary hybridization matrix. On the basis of our experience with cosmid 1F7, it was assumed that only strong hybridization signals clearly represented overlaps of contiguous DNA. Moderate hybridization signals were not recorded because of their ambiguity; they could have resulted either from a very short region of overlap or from cross-hybridization between noncontiguous copies of a repeated sequence.

To monitor the progress of the project, the map was assembled several times as the sampling-without-replacement hybridizations proceeded. This process identified clones that had hybridized to more than two probes, either because a clone containing an unidentified repeat had been mistakenly used as a probe, or because a clone containing one of the identified repeats had been intentionally used as a probe to saturate the map. Clones that hybridized to more than two probes were problematic because some of their apparent linkages had to be false. Nine such problem areas were detected and resolved by using one of the following two strategies. The first strategy was to identify another clone that mapped to a given problem area that either did not contain the problematic repeat or had the repeat at a distance from the ends of its insert. When such an alternative was identified, it was used as a probe and the results from the problematic probe were replaced with the new results in the binomial hybridization matrix. Five of the nine problem areas were resolved by employing this strategy. In the four remaining cases, no suitable alternative probes were identified. To resolve these problem areas, Southern blot analysis of restriction-enzyme-digested clones was used to distinguish between hybridization due to actual overlaps and hybridization due to shared repeated sequences. This strategy allowed deletion of repeat-generated hybridization data from the binomial matrix.

Cloning of repeated sequences: Because repeated sequences may play a role in the instability of the MAK1 chromosome, and because the isolation of repeated sequences would help identify repeat-free clones that could be used as probes in problematic regions of the map, three of the repeats on the MAK1 chromosome were cloned when analysis of the map became complicated. In each case, potential repeat-containing sub-
clones were identified via Southern analysis of cosmids that cross-hybridized moderately during the sampling-without-replacement experiments (data not shown). One of the repeats, Nht1, was first identified during cloning of the MAK1 gene. It was cloned from cosm id 1F7 and has been characterized in some detail; it is a putative class II transposable element 2.2 kb in length (Enkerli et al. 1997). The second repeat, named JE9, was alluded to earlier as it is present on cosmid 2H6. JE9 was subcloned as a 4-kb NtI restriction fragment from a cosm id (4B11) that hybridized moderately to 2H6. The third repeat, named HR1, was first identified during resolution of a problem area in the map. HR1 was subcloned as a 1.3-kb EcoRI restriction fragment from a cosm id (4A3) that hybridized moderately to cosmid 5B8. Neither JE9 nor HR1 has been characterized in detail; however, when used to probe CHEF-gel-separated chromosomal DNA of isolate 156-30-6, each hybridized exclusively to the MAK1 chromosome (data not shown). This result contrasts with the genomic distribution of Nht1; although most Nht1 copies are on the MAK1 chromosome, a few copies are present on some of the other chromosomes in the 156-30-6 genome (Enkerli et al. 1997). These three repeated sequences accounted for three of the nine problem areas in the map.

**Map assembly:** Once the problems caused by cross-hybridization were resolved, the final binary hybridization matrix was assembled into a map. The resulting map consisted of 33 probes organized into eight unordered contigs. Of the 442 clones in the chromosome-specific library, 232 are included in the map. The 210 remaining clones belong to the M clone collection and all, except for 4, are R clones. The map containing all 232 clones may be accessed at http://www.fungus.genetics.uga.edu:5080/ nhaem.html. All linkages in the map, except for two, are redundant (i.e., covered by >1 linking clone). DNA gel blot analysis supports the conclusion that the two nonredundant linking clones (2E2 and 5B6) contain inserts that are contiguous in the 156-30-6 genome (data not shown).

The complete map of the MAK1 chromosome was compressed into a minimal map by deleting redundant clones (Figure 2). This version of the map consists of all probes and just one linking clone between each pair of probes. In Figure 2 the eight unordered contigs generated by sampling without replacement are ordered on the basis of subsequent experiments (described below). To confirm the connection of the corresponding probes, each linking clone in the minimal map was used as a probe. In some cases, Southern blot analysis of restriction-enzyme-digested clones was also done to confirm connections. The clones to which each cloned repeated sequence hybridized are also indicated in Figure 2. Nht1 is present in at least six locations, JE9 in at least five locations, and HR1 in at least seven locations along the MAK1 chromosome.

Sampling without replacement selects against probes that overlap. However, as can be seen in Figure 2 some probes in the map do overlap (e.g., probes 1 and 30, and probes 26 and 32). In most cases this was due to the fact that while solving problem areas it became necessary to use some clones that had previously hybridized to probes as probes. In one case, however, overlapping probes resulted from the fact that a moderate hybridization signal generated by a very short overlap was mistakenly interpreted initially as being caused by cross-hybridization between repeated sequences.

At the conclusion of sampling without replacement, the end clones in each contig had hybridized only to a single probe. It was possible, therefore, that such clones overlapped with end clones from another contig. Consequently, single end clones or collections of end clones were used to probe the library to determine if they overlapped with other contig ends. None of them did.

The length of the map was estimated by assuming that each probe contains a 35-kb insert and that each pair of linked probes is separated by 8.8 kb (25% of 35 kb). These values are based on the measurement of 10 cosm id inserts via restriction digestion, and the fact that a probability calculation predicts that the space between probes is 25% of the average probe length (Hemmer 1989). The standard error of the average probe length is ±2 kb. Clones linked to a single probe (e.g., 1G12) were estimated to contribute 21.9 kb to the map because only 37.5% of their average length (35 kb) was estimated to overlap with a probe (1 − 0.25 = 0.75; 0.75/2 = 0.375). Pairs of overlapping probes were assumed to contribute 43.8 kb (35 + 8.8 kb) to the length of the map, or the same amount as 1 probe + 1 linking clone. The estimated sizes of the different contigs are indicated in Figure 2. The complete map is predicted to cover 1.499 Mb, or 94% of the 1.6-Mb MAK1 chromosome. The remaining 102 kb of the chromosome appears to be absent from the library. This corresponds to two to three missing probes (102/43.8 kb).

**Identifying contigs linked to chromosome ends:** To identify the contig ends that contain telomeric sequences or subtelomeric sequences (i.e., DNA that is immediately adjacent to a telomere), the library was hybridized to a chromosome-end-specific probe synthesized by extension of a primer containing three copies of the telomeric repeat from F. oxysporum. N. hamato-cocca MP VI isolate 156-30-6 genomic DNA was used as a template for this primer extension reaction. All 18 clones of the chromosome-specific library that hybridized to this probe were R clones. Four of these clones (1G12, 2C2, 4H3, and 5F5) are at one end of contig 1 (http://www.fungus.genetics.uga.edu:5080/nhaem.html), and one (1H9) is at one end of contig 8 (Figure 2). The remaining 13 clones were not linked to any probe used in the map. They appear, therefore, to belong to other chromosomes and apparently were segregated into the chromosome-specific library because of
cross-hybridization between telomeric and/or subtelomeric sequences. Restriction enzyme digestion and Southern blot analysis confirmed that cosmids 1G12, 2C2, 4H3, and 5F5 overlap with probe 11 and that cosmid 1H9 overlaps with probe 10. In addition, Southern analysis of 1H9 and genomic DNA from 156-30-6 supports the conclusion that 1H9 is not a chimera formed between unlinked subtelomeric DNA and the DNA linked to contig 8 (data not shown).

Closing gaps and ordering contigs: Two strategies were used to attempt closure of the seven gaps in the map. The first strategy is a PCR-based method that ne-
Three clones at the end of contig 7 (4H9, 2B2, and 1C7), probe 27 (1H2), and a clone linking probe 27 with its neighboring probe in contig 7 (2C6) were digested with EcoRI, separated by gel electrophoresis, and Southern blotted. The blot on the left was hybridized with the T7 end of 4H9 and the blot on the right was hybridized with the T3 end of 4H9. Molecular weight standards are indicated on the far left. (B) Physical organization of clones at the probe 27 end of contig 7 based on hybridization signals in A. T7 and T3 ends of clone 4H9 are indicated. The T3 end of cosmid 4H9 represents the terminus of the contig because the T3 probe hybridized only to itself. Dashed lines represent EcoRI sites that create a common 2.5-kb fragment in clones 2B2, 1C7, and 1H2, all of which hybridized to the T7 probe of 4H9.

As indicated in Figure 2, FR1 connects contigs 6 and 7; FR3 connects contigs 5 and 6, and FR13 connects contigs 7 and 8. Sequence analysis of FR1 revealed that contigs 6 and 7 actually overlap by 4 bp (GATC); the sequence of this overlap is the recognition sequence for MboI, the enzyme used to digest 156-30-6 genomic DNA during library construction (Covert et al. 1996).

The second strategy was used in an attempt to order the remaining four contigs in the map. In this approach, genomic DNA of N. haematococca MP VI isolate 156-30-6 was digested with a rare-cutting restriction enzyme,
Figure 5.—DNA gel blots identifying SwaI fragments bridging gaps in the map. Genomic DNA from isolate 156-30-6 was digested with SwaI, separated by CHEF electrophoresis, and blotted. The cosmids probe for each blot is indicated at the top of each lane. The blots probed with 2H2 and 5E2 came from the same gel. The blots probed with 5C3 and 4A3 came from a second gel. One lane from this gel is shown at right as an example of the SwaI digestion pattern. Molecular weight markers are indicated on the left of each panel in kilobases.

Figure 6.—Chromosome breakpoint mapping. The double arrow represents the MAK1 chromosome of isolate 156-30-6. The sizes of the different chromosomes used as probes are indicated on the left; their source isolates are in parentheses. Contigs or parts of contigs hybridizing to the chromosome of a given isolate are shown as horizontal dashed lines. Identified breakpoints are indicated with vertical dashed lines.

separated by CHEF electrophoresis, and probed with clones near the termini of the remaining contig ends. Probes that hybridized to equivalently sized DNA fragments were considered to be linked. Cosmids 2E9 and 5E2 both hybridized to a 70-kb NotI fragment, thus suggesting that the probe 32 end of contig 1 is linked to the 5E2 end of contig 2 (data not shown). This result was further confirmed by hybridization of cosmids 2H2 and 5E2 to a 100-kb SwaI fragment (Figure 5). Cosmids 5C3 and 4A3 both hybridized to a 160-kb SwaI fragment, thus suggesting that the 5C3 end of contig 2 is linked to the probe 22 end of contig 3 (Figure 5). Finally, cosmids 3E2 and 2B5 both hybridized to a 50-kb NotI fragment (data not shown), thus suggesting that the probe 4 end of contig 3 is linked to the clone 2B5 end of contig 4.

In summary, these approaches allowed closure of six gaps in the map and ordered the eight small contigs into the two large contigs (A and B) shown in Figure 2. Including the data from the PCR-based gap closure, the estimated total length of the map is 1.52 Mb or 95% of the MAK1 chromosome. The length of the gaps closed by NotI and SwaI restriction analysis is not included in this length estimate because the extent of overlap between the terminal clones and the hybridizing restriction fragments is not known.

Breakpoint mapping: While investigating the inheritance of phytoalexin detoxification in N. haematococca MP VI, Miao and VanEtten (1992) crossed two isolates containing the 1.6-Mb MAK1 chromosome (isolates 156-30-6 and 230-30-6). Truncated forms of the chromosome were found in many of the progeny of this cross (Miao et al. 1991). To identify the points of chromosome breakage, we probed the MAK1-chromosome-specific library with truncated forms of the MAK1 chromosome from four of these progeny. The signals from each hybridization experiment were compared to the contig map of the MAK1 chromosome (Figure 6). The hybridization data from the 1.0-Mb chromosomes in isolates 272-1-1 and 272-1-5 were identical: contigs 3, 4, and 6–8 were lost. Probing with these two chromosomes identified two breakpoints flanking an internal deletion; one maps to the gap closed by the 160-kb SwaI fragment linking contigs 2 and 3, and one maps to the unclosed gap between contigs A and B. Probing with these two chromosomes also identified a terminal deletion that maps to cosmids 2F11. Probing with the 1.5-Mb derivative of isolate 272-6-2 identified a breakpoint very close to one end of contig B; cosmids 4G2 (Figure 2) was the last clone to hybridize to this chromosome. The 1.0-Mb derivative of isolate 272-6-1 hybridized to all of contig B and to contigs 3 and 4. Its breakpoint falls into the gap closed by the 160-kb SwaI fragment linking contigs 2 and 3. In all cases, the hybridization data were consistent with the Mak and Pda phenotypes of the source isolates; e.g., isolates that lack Mak and Pda activities did not hybridize to the contig that contains MAK1 and PDA6-1 (Table 1; Figure 6).

DISCUSSION

As a first step in our detailed structural analysis of a CD chromosome, a physical map of the MAK1 chromosome in N. haematococca MP VI was constructed by relying upon DNA-DNA hybridization to detect linkages between overlapping clones. Despite the complications introduced by the many dispersed, repeated sequences...
Physical Map of a CD Chromosome

on the MAK1 chromosome, it was valuable to rely on this approach because we gained information on the number and position of the dispersed repeats as the map developed. We anticipate that knowledge of these elements will be relevant to future studies designed to examine the relationship between the repeats and the instability of the MAK1 chromosome during sexual reproduction. At an estimated length of 1.52 Mb, the MAK1 chromosome map covers 95% of the 1.6-Mb chromosome. This is similar to the estimated coverage of other physical maps, such as the Aspergillus nidulans genome map (80–100% coverage/ chromosome; Pradé et al. 1997) and the Candida albicans chromosome 7 map (100% coverage; Chibana et al. 1998), which are derived primarily from hybridization data. Restriction enzyme fingerprinting of large-insert clones can also be used to construct physical maps (Taylor et al. 1996; Marr et al. 1997) but this method does not simultaneously gather information on the pattern of repeats on a chromosome. This approach was recently combined with DNA-DNA hybridization to rapidly construct a robust map covering 95% of chromosome 7 in Magnaporthe grisea (Zhu et al. 1999). A more complete understanding of the chromosome structure and function could of course be obtained from the complete nucleotide sequence of the MAK1 chromosome. If the resources for such a project should become available, the cosmids comprising the minimal map (Figure 2) could readily serve as an ordered template for the sequencing effort.

To anchor the ends of the map to the actual chromosome ends, a probe extended from telomeric repeats was hybridized to the MAK1-chromosome-specific library. Our reasoning was that such a probe would identify clones containing telomeric repeats and/or subtelomeric sequences. In Cladosporium fulvum, DNA homologous to subtelomeric sequences is also present at nonterminal locations in the genome (Colman et al. 1993). If a similar situation existed on the MAK1 chromosome, clones from multiple locations in the map would hybridize to the telomere-derived probe. However, only two locations in the map hybridized to this probe and both of them were at contig ends. This strongly suggests that the identified contig ends are adjacent to the telomeres. Four different clones represent the chromosome end identified in contig 1. This level of redundancy gives us confidence that the apparent linkage between contig 1 and a telomere is real. In contrast, only cosmid 1H9 represents the chromosome end identified in contig 8. Our confidence in this apparent linkage was raised by the finding that 1H9 appears to contain a contiguous piece of N. haematococca MP VI DNA.

After completion of the sampling-without-replacement protocol, the eight unordered contigs in the map were separated by seven gaps estimated to total 102 kb. Successful closure of three gaps by PCR (totaling ~23 kb) was particularly useful because this approach not only identified linkages between contigs, but also supplemented the library with the missing DNA sequences. PCR presumably failed to close the four remaining gaps in the map because their length exceeded the range of long-distance PCR, although at least some should have been within this upper limit [(102 – 23)/4 = 20 kb]. Rare-cutting restriction enzyme mapping allowed orientation of six additional contig ends so that there is only one uncharacterized gap in the final map. This contig-ordering technique was advantageous relative to the PCR-based technique because it could potentially detect linkages between contigs even if they were separated by several hundred kilobases. The overall utility of this approach, however, was limited by the fact that it does not readily lead to isolation of the DNA bridging the gap.

Thirty-eight percent of the clones included in the minimal map are known to contain repeated DNA sequences (i.e., they are R clones or contain Nht1, JE9, and/or HR1). Although they are dispersed throughout the chromosome, these repeats appear to be concentrated in particular areas of the MAK1 chromosome. For example, contig 1 seems to be especially enriched in repeated sequences compared to contigs 5 or 6 (Figure 2). Additional repeats that accounted for six problem locations in the MAK1 chromosome map were not cloned during map construction; thus, the quantity of clones containing repeats is probably >38%. The successful construction of a linear map via a hybridization-dependent methodology demonstrates that the many repeated sequences on the MAK1 chromosome are embedded within long stretches of single-copy DNA specific to this CD chromosome. The MAK1 chromosome, therefore, is not an accumulation of sequences derived from other chromosomes but represents a unique genetic entity in the N. haematococca MP VI genome.

Two of the repeated sequences on the MAK1 chromosome, JE9 and HR1, are not present on other chromosomes in the 156-30-6 genome. The supernumerary chromosomes in two other plant pathogens are also characterized by distinctive repeated DNA patterns. In certain isolates of Colletotrichum gloeosporioides, a 1.2-Mb supernumerary chromosome lacks two repeated sequences found on other chromosomes in the genome (Masel et al. 1993, 1996). One of these repeats appears to be a non-LTR, LINE-like retrotransposon (He et al. 1996). In Cochliobolus carbonum a putative class II transposon, Fcc1, is present on most of the chromosomes but it appears to be especially common on a 3.5-Mb chromosome composed largely of supernumerary DNA (Ahn and Walton 1996; Panaccione et al. 1996). The distinctive signatures of repeated sequences on these supernumerary chromosomes suggest that they may have unique evolutionary histories relative to the essential portions of their resident genomes. The distribution of JE9 and HR1 in the genomes of many N. haematococca MP VI field isolates will need to be examined to see if...
this idea can be substantiated in this particular species. Repeated sequences interspersed between blocks of single-copy DNA were also detected during physical mapping of the A. nidulans genome, but the frequency and location of individual repeats were not characterized (Prade et al. 1997).

In addition to providing information on the quantity and location of repeated DNA on the MAK1 chromosome, the physical map of this chromosome was used to characterize breakpoints generated during sexual reproduction. The truncated chromosomes in isolates 272-1 and 272-1-5 appear to have been formed by two events: an internal deletion (contigs 3 and 4) and a terminal deletion (contigs 6-8). The truncated chromosomes in 272-6-1 and 272-6-2 were formed more simply by terminal deletions with different breakpoints. The terminal deletions in the chromosomes from 272-1, 272-1-5, and 272-6-2 map to positions covered by cloned DNA. Fine structure mapping in these regions will be conducted to identify the sequences at the breakpoints and thus to determine if dispersed repeats could be mediating these truncation events. The other mapped breakpoints fall into regions that have not been cloned. The fact that at least two different deletion events mapped to the gap between contigs 2 and 3 provides some crude evidence for a recombination hotspot in this region. It will be necessary, however, to isolate the missing DNA sequences in this region before a detailed analysis of the breakpoints in this location can be undertaken. Because contig 5 is present in all four truncated chromosomes, it is possible that the centromere is in this region. However, centromeres are frequently characterized by the presence of repeated sequences (Cen
tola and Carbon 1994; Eichler 1999) and contig 5 does not include any R clones, i.e., clones with DNA present on other chromosomes. Alternatively, the centromere could lie in the gap between contigs 4 and 5. The clones in contig 4 flanking this gap are R clones, so they might signal the beginning of a region containing centromeric repeats.

The meiotic products from cross 272 were collected as complete tetrads (Miao et al. 1991; Miao and VanEtten 1992). As a consequence, it is possible to infer when the MAK1 chromosome was truncated during sexual reproduction. Isolates 272-1 and 272-1-5 appear to contain structurally identical versions of the MAK1 chromosome, but because their female fertility differs they are thought to represent two different meiotic products of ascus 1 (V. Miao, personal communication). The simplest interpretation of this finding is that the deletions occurred before DNA replication and thus before meiosis. Alternatively, the relatively complicated events that created these versions of the chromosome (i.e., two deletions) occurred in identical locations, on two different DNA molecules. This seems less likely than the former. In isolates from ascus 6, the size of the MAK1 chromosome in each meiotic product is different (Miao et al. 1991). This variety indicates that at least some of the events forming the derivatives in ascus 6 took place after DNA replication. Our data support this idea further by demonstrating directly that the derivatives of the MAK1 chromosome in isolates 272-6-1 and 272-6-2 are missing completely different portions of the chromosome. In short, it appears that the MAK1 chromosome can be truncated at different stages during sexual reproduction. Analysis of additional tetrads and the types of chromosome rearrangements within them should give further insight into the timing of these events.

The mapping of breakpoints on the MAK1 chromosome is also a first step toward determining if there is a relationship between chromosome structure and chromosome instability in N. haematococca MP VI. In the tetrads collected from cross 272, truncated chromosomes of the same size were generated in more than one ascus (Miao et al. 1991). This finding suggested that certain locations on the MAK1 chromosome are particularly susceptible to breakage (Miao et al. 1991). Dispersed repeated sequences on the MAK1 chromosome might mediate specific patterns of truncation by serving as sites for reciprocal recombination, single-strand annealing, or replication slippage (Klein 1995). Deletions due to reciprocal recombination could result from unequal exchange between direct repeats on sister chromatids or on homologs, or from exchange between direct repeats on the same chromatid (Klein 1995). Currently, it appears unlikely that unequal exchange between sister chromatids or homologs accounts for the deletions recovered from cross 272 because chromosome truncation was always observed without concomitant chromosome extension in a sibling from the same tetrad (Miao et al. 1991). However, in a subsequent cross between two isolates carrying different truncated forms of the chromosome (272-6-1 and 272-6-2; Figure 6; Miao et al. 1991), changes in the size of the MAK1 chromosome appear to have resulted from reciprocal recombination between homologs of unequal length.

Dispersed repeated sequences are not the only structures that might contribute to the instability of the MAK1 chromosome. Meiotic recombination hotspots, regions of unusually high recombination frequency, could also be involved. Recombination hotspots have been identified in fungi, plants, nematodes, mice, and humans (Lichten and Goldman 1995). The recombination hotspots in Saccharomyces cerevisiae and Schizosaccharomyces pombe are the most well studied and are characterized by chromatin, such as intergenic-promoter-containing DNA or protein-binding DNA, which is likely to be structurally accessible to proteins involved in meiotic recombination (Nicolas 1998). In the future, a detailed analysis of the DNA sequences located at breakpoints should allow us to determine what chromosome structures, if any, are contributing to the instability of the MAK1 chromosome in N. haematococca MP VI.
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