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Cloning and Characterization of Centromeric DNA from *Neurospora crassa*

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The centromere locus from linkage group VII of *Neurospora crassa* has been cloned, characterized, and physically mapped. The centromeric DNA is contained within a 450-kb region that is recombination deficient, A+T-rich, and contains repetitive sequences. Repetitive sequences from within this region hybridize to a family of repeats located at or near centromeres in all seven linkage groups of *N. crassa*. Genomic Southern blots and sequence analysis of these repeats revealed a unique centromere structure containing a divergent family of centromere-specific repeats. The predominantly transitional differences between copies of the centromere-specific sequence repeats and their high A+T content suggest that their divergence was mediated by repeat-induced point (RIP) mutations.

Centromere analysis is being carried out on several evolutionarily distinct organisms concurrently. Collectively, this research has shown that the centromere is an intricate machine that orchestrates proper segregation of chromosomes during cell division. This structure captures and moves along microtubules in a spatially and temporally regulated manner during mitosis. Although centromere function is highly conserved, centromere structure is variable, increasing in size roughly in proportion to the complexity of the organism in which it is studied (5, 12).

Mammalian cells are a rich model system in which to study centromere structure because of the intricate structure and large size of centromeres in these organisms. The centromeric DNA of these cells, which can span megabases, is made up of tandemly arranged, species-specific sequence repeats (28, 42). The DNA is organized into a highly ordered chromatin structure, in the form of a trilaminar disk, known as the kinetochore (36, 37). The complexity of these structures, however, also increases the difficulty of molecular characterization.

In contrast, the centromere from the budding yeast *Saccharomyces cerevisiae* is well characterized at the molecular level. A functional *S. cerevisiae* centromere is specified in *cis* on a 125-bp DNA sequence (*CEN*) (12, 13). The *CEN* locus is organized into three functionally distinct centromere DNA elements (CDEs). CDE I and III are short sequences, 8 and 25 bp, respectively, which are sequence-specific protein-binding sites. CDE I and III flank an A+T-rich, 78- to 86-bp central sequence, known as CDE II. Biochemical purification and in vitro functional studies have identified at least four different proteins which are involved in centromere function in *S. cerevisiae* (2, 4, 9, 21).

The centromere of the fission yeast *Schizosaccharomyces pombe* is intermediate in size and complexity between the centromeres of *S. cerevisiae* and mammals. The centromeric DNA on the three chromosomes of *S. pombe* spans 38, 80, and 100 kb in *cenI*, *cenII*, and *cenIII*, respectively (3, 19, 30). On all three chromosomes of *S. pombe*, the centromeric DNA is organized into two large inverted repeat sequences that border an A+T-rich central core. The sizes of the inverted repeats vary between chromosomes and between strains because of differences in the arrangement and number of subrepeats they contain.

In an effort to bridge the information gap between the centromere model systems described above, we have characterized a centromere from the multicellular filamentous ascomycete *Neurospora crassa*. This organism was chosen because it shares the small genome and chromosome size as well as the ease of genetic and biochemical manipulation of yeasts, but, like animal cells, *N. crassa* chromosomes, within the ascus, have been shown to condense in both mitosis and meiosis. The condensed chromosomes contain heterochromatin-like chromomeres, some of which may be associated with centromeres (24, 34, 43). To isolate an *N. crassa* centromere, we constructed a yeast artificial chromosome (YAC) library containing 140- to 260-kb cloned segments of *N. crassa* genomic DNA and isolated a set of contiguous clones (contig) spanning the centromere of linkage group VII (lgVII). A 450-kb region within this contig is A+T rich and recombination deficient and contains centromere-specific DNA sequence repeats.

MATERIALS AND METHODS

Strains, media, and enzymes. *Escherichia coli* WM1100 *araD139* Δ(*ara leu*)*7697* Δ(*lac*)*74 galU galK hsdR2 strA mcrA mcrB1* Δ(*srl-recA*)*306*::Tn*10* purchased from Bio-Rad, Inc. (Richmond, Calif.) and DB6656 *lacZ624*(Am) *trp-49*(Am) *pyrF79*::Mu *rpsL179 hsdR27* (1) were used for electroporation transformations (16) and preparation of plasmid DNAs. *S. cerevisiae* AB1380 *MAT***a** *ade2-1 can1-100 lys2-1 trp1 ura3 his5* [*psi*$^+$] (8) was used as a host for the preparation of the YAC library. *N. crassa* wild-type strain 74-OR23-1A (Fungal Genetics Stock Center 987 [74A]) was used as a source of genomic DNA for YAC library preparation and genomic mapping. Standard bacterial (23), yeast (41), and *N. crassa* (45) growth media were used. Restriction enzymes, T4 DNA ligase, and appropriate reaction buffers were purchased from New England BioLabs, Inc. (Beverly, Mass.). Reaction conditions used were specified by the

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vendor. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim (Indianapolis, Ind.).

DNA preparation, gel electrophoresis, colony hybridization, and Southern blot analysis. Standard protocols were used as described previously (23) except where noted. *N. crassa* and yeast DNA preparations were done as described earlier (15, 26).

YAC library screening. A detailed paper describing construction of the *N. crassa* YAC library will be published elsewhere. The library was prepared with the pYAC-4 vector and with *S. cerevisiae* AB1380 as a host (8). The 2,280-clone library was ordered into microtiter dish wells and is deposited with the Fungal Genetics Stock Center (University of Kansas). Yeast colony hybridization for YAC library screening was done as previously described (6).

YAC restriction mapping and contig building. Yeast clones containing artificial chromosomes were grown up in selective media, and agarose plugs containing genomic DNA were prepared (44). Plugs were treated with phenylmethylsulfonyl fluoride (United States Biochemical Corp., Cleveland, Ohio) (44), and DNA within the plug was partially digested with restriction enzymes as follows. Twenty microliters of plug was melted at 68°C for 5 min. The plug was cooled to 37°C, and a 1/10 volume of prewarmed restriction buffer (10× concentrate) was added. Reactions with prewarmed restriction enzyme (0.2 and 1.0 U/μl) were carried out on each sample at 37°C for 30 min. Digests were fractionated on a Bio-Rad contour-clamped homogenous electric field (CHEF) DR II apparatus (46) in a 1% agarose gel at 6.0 V/cm, with an 8-s switch time, in 0.5× Tris-borate-EDTA for 18 h at 14°C. Gels were blotted to Sureblot membranes (Oncor, Inc., Gaithersburg, Md.) and hybridized with a YAC vector end-specific probe. Duplicate samples were run, blotted, and hybridized with a probe specific for the opposite YAC arm. Mapping data from the two hybridizations were averaged to generate the final restriction map. Maps for clones from a given step in the walk were compared and overlapped by visual examinations to generate contigs. Contig overlaps between steps in the walk were confirmed by cross-hybridization on Southern blots.

Hybridization probes specific for the left and right arms of the pYAC-4 vector. Gel-purified 1.7-kb *Bam*HI-*Pvu*II fragment from pBR322 hybridizes specifically to the *URA3*-containing YAC vector arm (*URA3* arm). Intact pBluescript (Stratagene, La Jolla, Calif.) hybridizes specifically to the *TRP1*-containing YAC vector arm (*TRP1* arm).

Isolation of terminal restriction fragments from cloned DNA inserts in YAC clones. Terminal fragments were isolated from the cloned insert nearest the *TRP1* ends of YAC clones 24-3-G, 15-6-H, and 19-5-B by plasmid rescue in *E. coli* as described elsewhere (18), with the following modifications: plasmid rescues were done by using *Pac*I genomic digests of YAC clones, and the subsequent ligation reaction products were used to transform *E. coli* by electroporation. There are two *Pac*I sites on pYAC-4, each occurring near the junction of the *Tetrahymena* telomere regions in each arm of the YAC. Digestion of a YAC clone with *Pac*I results in a DNA fragment containing *E. coli* Amp^r and *ori* and insert DNA up to the first *Pac*I site in the *TRP1* end of the insert of the clone; this fragment can be circularized and cloned in *E. coli* as a plasmid.

Isolation of terminal fragments of insert DNA from the *URA3* arm of YAC clones was carried out as follows. The yeast *URA3* gene complements a *pyrF* mutation in *E. coli* (1). *URA3* arm terminal fragments of insert DNA were subcloned into *E. coli* by ligating *Xho*I restriction fragments from the YAC into pBluescript and transforming a *pyrF E. coli* strain and then selecting for complementation of *pyrF* by a hybrid plasmid containing the *URA3* gene and insert DNA up to the first *Xho*I site in the *URA3* end of the insert. One microgram of YAC 12-10-H genomic DNA and 1 μg of pBluescript DNA were digested separately with 10 U of *Xho*I at 37°C for 3 h; calf intestinal alkaline phosphatase (0.5 U) was added to the pBluescript digest, and the reaction mixture was incubated for an additional 30 min. Both reaction mixtures were extracted with phenol-$CHCl_3$, and the DNA was precipitated by adding 2 volumes of ethanol. The genomic DNA (100 ng) and pBluescript (100 ng) *Xho*I fragments were ligated in a 20-μl reaction mixture with T4 DNA ligase. The ligation reaction product (1 μl) was electroporated into *E. coli* DB6656, and cells were plated on minimal medium supplemented with 2 g of Casamino Acids (M9CA media) per liter and 50 μg of ampicillin per ml. Plasmid DNAs from five ampicillin-resistant *URA*$^+$ colonies were prepared and digested with *Xho*I. All five plasmids had identical *Xho*I restriction patterns, containing a 2.9-kb fragment (pBluescript) and a 3.8-kb fragment whose size matched that of the terminal *Xho*I fragment from the insert of YAC 12-10-H. An 800-bp *Xho*I-*Eco*RI restriction fragment from the rescue plasmid hybridized to the terminal 800-bp *Eco*RI-*Xho*I fragment in the insert of YAC 12-10-H on genomic Southern blots of the YAC clone.

Calculation of A+T content within the YAC contig region. Average A+T percent across the 450-kb centromere region was derived from the following equation: number of *Pac*I sites/total length of the DNA in base pairs = $(A+T\%/200)^8$.

Quantitative Southern blot analysis. Aliquots of genomic DNA (2 μg) were digested in three separate reactions with 15 U of *Eco*RI, *Hin*dIII, or *Sal*I. The reaction mixtures were split into two fractions and each half was loaded on a single 0.7% chromosomal-grade agarose (Bio-Rad, Inc.) gel (see Fig. 5). The gel was electrophoresed in a Bio-Rad CHEF DR II at 6 V/cm and 14°C for 14 h in 0.5× Tris-borate-EDTA with a constant pulse time of 1.5 s. The gel was blotted to a Sureblot membrane (Oncor, Inc.) and the membrane was baked for 1 h in a vacuum oven, cut into two pieces between the two sets of identically loaded samples, and hybridized as described below.

Equal masses of both the 800-bp end-rescue fragment from YAC 12-10-H and of an 800-bp *Bgl*II-*Hin*dIII fragment from the *N. crassa qa-2* region (38) were labelled separately with [α-^{32}P]dCTP by random priming by using the Prime-It labelling kit (Stratagene, Inc.) as specified by the vendor. The probes were labelled to nearly equal specific activities (800-bp fragment, 8.9×10^5 cpm/ng; *qa-2* fragment, 9.4×10^5 cpm/ng). One membrane was hybridized with 1×10^6 cpm/ml of the 800-bp fragment. The second was hybridized simultaneously with an equal volume of 800-bp and *qa-2* fragments to a total of 1×10^6 cpm/ml. Membranes were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate at 65°C for 10 min and twice in 0.5× SSC at 65°C for 10 min. The filters were autoradiographed for 16 h with an enhancing screen. Signal intensities of bands on the autoradiograph were determined by densitometry with an LKB Ultroscan densitometer (LKB, Bromma, Sweden).

Rescue of internal DNA segments from YAC clones 12-10-H and 10-8-H. A vector that could be used for homologous site-directed integration of an *E. coli* selectable marker and *ori* flanked by several restriction sites into YAC clones 12-10-H and 10-8-H was constructed as follows. The 800-bp *Eco*RI-*Xho*I restriction fragment from the end of the *Neu-*

rospora DNA insert in YAC 12-10-H and the 900-bp *Sal*I-*Cla*I restriction fragment that contains the *S. cerevisiae SUP4* gene from pYAC-4 were ligated into *Eco*RI-*Sal*I-digested pBluescript. The resultant construct was named pKS800 (see Fig. 6A).

One microgram of pKS800 was cut at a unique *Bgl*II site within the 800-bp terminal fragment from YAC 12-10-H and transformed into yeast strains AB1380/YAC 12-10-H and AB1380/YAC 10-8-H by spheroplast transformation as previously described (7). Transformants were selected by *SUP4* suppression of the *ade2* mutation in AB1380 on minimal salts-dextrose medium supplemented with Casamino Acids. Flanking DNA sequences from YAC clones into which pKS800 had integrated were rescued in *E. coli* as described above (see the diagram in Fig. 6).

RFLP mapping. Restriction fragment length polymorphism (RFLP) mapping of restriction fragments containing centromere repeats was carried out by using a set of 38 segregants from ordered asci derived from a cross of the Oak Ridge-derived parent strain multicent-2-a and the Mauriceville-1c-A parental strain (27). DNA from clones of these 38 segregants has been widely distributed, and segregation data on RFLPs from over 100 independent, cloned DNA fragments from the *Neurospora* genome have been determined. These data have been compared and ordered into a high-density RFLP map that covers the entire genome (25). The genomic location of unmapped cloned *N. crassa* DNA fragments is determined by (i) identification of an RFLP between the two parental strains to which the DNA fragment hybridizes, (ii) determination of the segregation pattern of the RFLP in DNA from the 38 segregants described above, and (iii) comparison of the pattern obtained to the extensive RFLP maps compiled for these segregants by visual inspection. DNA from the segregants was kindly provided by Tom Schmidhouser (Southern Illinois University). Genomic DNAs from the segregants and parental strains were digested with either *Pac*I or *Kpn*I and electrophoresed on CHEF gels as described in the section on quantitative Southern analysis. Gels were blotted to membranes and hybridized with either gel-purified 3.2-kb or 800-bp terminal fragments from the cloned insert in YAC 12-10-H. Repetitive probes were used for this analysis; therefore, several bands appeared for a given parent and true allelic partners could not be scored for a given RFLP. Since an RFLP for a given location in the genome could be identified by the presence of a band on a genomic Southern blot of restriction enzyme-digested DNA from one parent and the absence of a band in digested DNA from the other parent, the presence of a given repeat fragment in one parental type was considered allelic to the absence of the same-sized band in the other parent. Centromere linkage was determined by comparison of segregation data of repetitive bands with previously mapped centromere-linked markers. Two autoradiographs, 16 h without an intensifying screen and 16 h with two intensifying screens, were taken and scored to accurately determine the RFLPs from a given Southern hybridization. Segregation data were submitted to the Fungal Genetics Stock Center for inclusion in the RFLP map of the *N. crassa* genome.

DNA sequencing and sequence analysis. DNA fragments were subcloned into pBluescript and were sequenced with the Sequenase kit (United States Biochemical Corp.) The DNA inserts were sequenced in both directions by using the forward and reverse primers supplied with the kit according to instructions provided by the vendor. Sequence analysis was done with the Pileup program from the Genetics Computer Group software package, using the suggested defaults of the program. The alignment of bases in the R2 repeat at positions 143 to 159 was done by visual inspection.

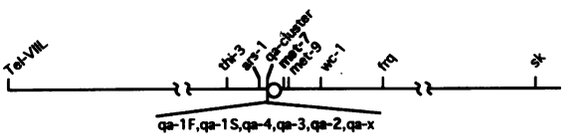

FIG. 1. Partial genetic map of lgVII showing the centromere and nearby genes. This map is derived from references 33 and 35, which contain full descriptions of marker genes. Locations of *ars-1* and the *qa* cluster relative to *cenVII* (circle) were determined in this work. Recombination frequencies vary widely between strains of *N. crassa* (35); therefore, the relative positions of markers are approximate and no scale is given.

RESULTS

Chromosome walking in the centromere region of lgVII. A partial genetic map of lgVII is shown in Fig. 1. A chromosome walk through the centromere region was initiated between the two cloned, centromere-linked genes *qa-2*$^+$ and *met-7*$^+$ (14, 38). *met-7*$^+$ is located on the right arm of lgVII, <1 centimorgan (cM) away from the centromere (35). *qa-2*$^+$ is also <1 cM away from the centromere, although its position relative to the centromere has not been determined, since no recombination between *qa-2* and *cenVII* has been found in several hundred scored asci (35).

A 2,200-clone YAC library of *N. crassa* genomic DNA was constructed. The library contains 8.8 genome equivalents; YAC clones range in size from 140 to 260 kb and have an average insert size of 180 kb. The YAC library was probed with a 2.1-kb *Hin*dIII-*Bam*HI DNA fragment that contains the *qa-2*$^+$ coding region and separately with two tandem 1.5-kb *Bam*HI DNA fragments that contain the *met-7*$^+$ coding region. Nine YAC clones hybridized to the *qa-2*$^+$ hybridization probe, and five YAC clones hybridized to the *met-7*$^+$ hybridization probes. The YAC clones were physically mapped with the restriction endonucleases *Not*I, *Asc*I, and *Sfi*I. Restriction maps of the *qa-2*$^+$- and *met-7*$^+$-containing YAC clones were overlapped into contigs by visual inspection. The *qa-2*$^+$- and *met-7*$^+$-containing YAC contigs were 300 and 245 kb long, respectively. Restriction maps of representative YAC clones that span the entire length of each of these contigs are shown in Fig. 2A.

No single YAC clone hybridized to both the *qa-2*$^+$ and *met-7*$^+$ hybridization probes, nor did the restriction maps of the *qa-2*$^+$- and *met-7*$^+$-containing contigs overlap. A chromosome walk between the contigs was therefore initiated. Both contigs were physically mapped with the restriction endonuclease *Pac*I. *Pac*I, which cleaves at an 8-bp recognition sequence that contains only AT base pairs, cleaves with dramatically increasing frequency on one end of both the *qa-2*$^+$- and *met-7*$^+$-containing contigs (Fig. 2). The average frequency of *Pac*I cleavage should increase in proportion to the average A+T content of the DNA substrate. Since A+T-rich DNA is characteristic of centromeric DNA in many organisms, chromosome walks were initiated in the direction of the contig ends that contained the highest frequency of *Pac*I sites.

The terminal 800 bp of insert DNA adjacent to the *URA3* arm of YAC 12-10-H (Fig. 2) was subcloned into *E. coli*. The *Xho*I fragment containing the terminal 800 bp of insert and *URA3* from the YAC arm was subcloned into pBluescript. The desired plasmid was obtained by selecting for comple-

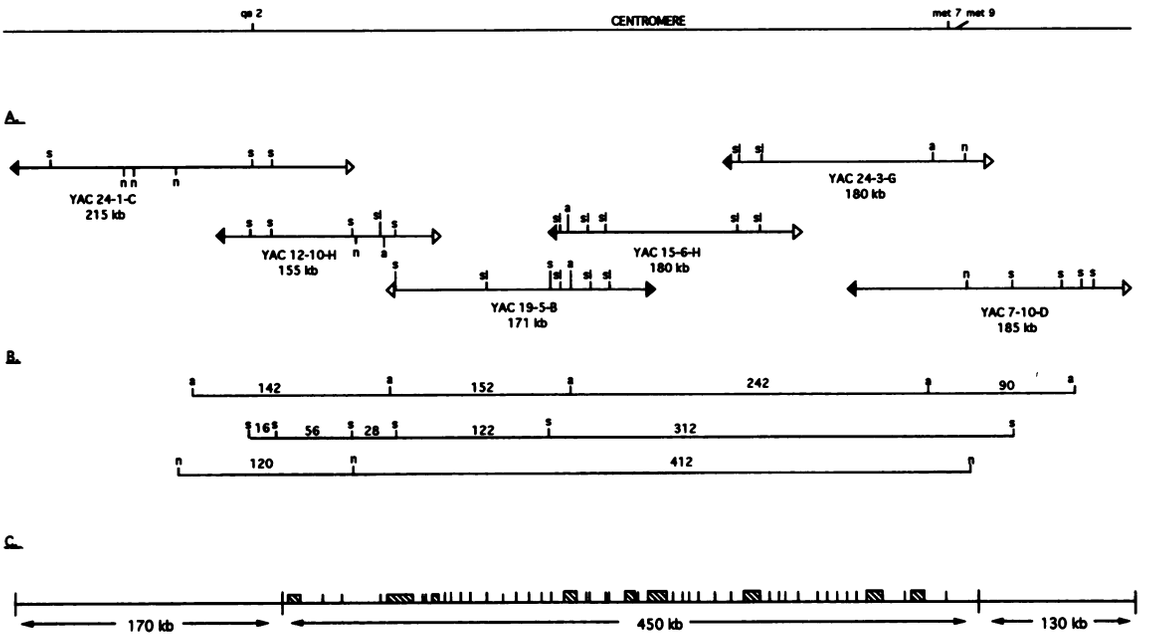

FIG. 2. Diagrammatic summary of the chromosome walk through the *cenVII* region. The relative locations of the centromere and the *qa-2*, *met-7*, and *met-9* genes are shown at the top of the figure. (A) Physical map of selected YAC clones spanning the centromere region of lgVII. Filled arrowheads represent *TRP1*-containing arms; open arrowheads represent *URA3*-containing arms. *Asc*I sites were not mapped on YAC clones 24-1-C and 7-10-D. (B) Physical map of the *cenVII* region derived directly from genomic Southern blots of restricted *N. crassa* genomic DNA. Physical distances between sites are in kilobases. The restriction enzymes used for analysis were as follows: a, *Asc*I; n, *Not*I; s, *Sfi*I; and sl, *Sal*I (*Sal*I sites in the centromere distal regions of YAC 12-10-H and 24-3-G were mapped but are not shown). The 56- and 28-kb *Sfi*I restriction fragments were not mapped from genomic Southern blots. These fragments were mapped only on the YAC clones but were included in the genomic map for continuity. (C) *Pac*I restriction map of YAC clones shown in panel A. Individual sites (vertical bars) and clusters of sites which could not be resolved (boxes) are shown. The length of the *Pac*I island and distances from the terminal *Pac*I sites to the ends of the YAC contig are indicated. A minimum number of sites are shown.

mentation of an *E. coli pyrF* mutant with the *S. cerevisiae URA3* gene (1). The YAC library was probed with the 800-bp *Xho*I-*Eco*RI terminal fragment, and 7% of the YAC clones hybridized to the probe. The 800-bp terminal fragment was hybridized to *N. crassa* genomic Southern blots and shown to be repetitive (see the discussion below) and thus could not be used as a hybridization probe for chromosome walking. The terminal 1.2-kb *Pac*I-*Eco*RI restriction fragment of insert DNA from the *TRP1* arm of YAC 24-3-G (Fig. 2) was rescued into *E. coli* (see Materials and Methods). This fragment hybridized strongly to single *Eco*RI and *Hin*dIII fragments in *N. crassa* genomic Southern hybridizations, although weak hybridization to three additional bands was seen (data not shown). The YAC library was also probed with this 1.2-kb terminal fragment. YAC clones 15-6-H and 5-12-E hybridized strongly to this probe.

To establish continuity among the YAC clones derived from this region, a restriction map of the *N. crassa* genome was generated. Southern blots of *N. crassa* genomic DNA cleaved with *Asc*I, *Sfi*I, and *Not*I were hybridized separately with the 2.1-kb *Hin*dIII-*Bam*HI DNA fragment that contains the *qa-2*$^+$ coding region, the two tandem 1.5-kb *Bam*HI DNA fragments that contain the *met-7*$^+$ coding region, and the terminal fragments from the inserts of YAC clones 12-10-H and 24-3-G described above. The *qa-2*$^+$ probe hybridized to a 120-kb *Not*I fragment (Fig. 3, panel A). The *met-7*$^+$ probe and the terminal probes from the inserts of YAC 12-10-H and YAC 24-3-G all hybridized to a 412-kb *Not*I fragment (Fig. 3, panels B, D, and E). Since the *qa-2*$^+$ probe and the YAC 12-10-H terminal probe are separated by

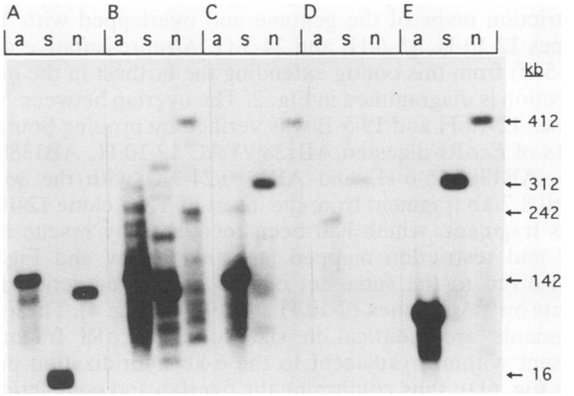

FIG. 3. Physical mapping of *N. crassa* genomic DNA in the *cenVII* region. Southern blots of *N. crassa* genomic DNA cleaved with the restriction enzymes *Asc*I, *Sfi*I, and *Not*I (lanes a, s, and n, respectively) hybridized with five restriction fragments from the *cenVII* region (panels A to E) are shown. The probes used were as follows: a 2.1-kb *Hin*dIII-*Bam*HI restriction fragment from the *qa-2* gene (A); an 8.2-kb *Cla*I-*Eco*RI terminal insert fragment from the *URA3* proximal end of YAC 12-10-H (B); a 4.8-kb *Pac*I-*Eco*RI terminal insert fragment from the *TRP1* proximal end of YAC 15-6-H (C); 1.2-kb *Pac*I-*Eco*RI terminal insert fragment from the *TRP1* proximal end of YAC 24-3-G (D); tandem 1.5-kb *Bam*HI fragments from the *met-7* gene (E). The numbered arrows on the right indicate fragment lengths.

one *Not*I site on the restriction map of the *qa-2*$^+$-containing contig, these data are consistent with the *Not*I restriction map in Fig. 2B. The locations of the *Asc*I and *Sfi*I restriction sites in this region were determined in the same manner. A restriction map of the genomic DNA from this region is shown in Fig. 2B. Hybridization probes derived from YAC clone 12-10-H contained repetitive DNA sequences and thus hybridized to multiple restriction fragments in the *N. crassa* genome (Fig. 3, panel B). The darkest hybridizing restriction fragments were assumed to be derived from the *cenVII* region (see the quantitative Southern blot analysis section below) in all cases, except for the 412-kb *Not*I band that hybridized weakly to the 12-10-H terminal probe (lane B1) but fits logically into the map shown above. The weak signal from this fragment is most probably due to preferential degradation or mechanical shearing of high-molecular-weight DNA during the cleavage reactions.

YAC clones 15-6-H and 5-12-E, which hybridized to the terminal probe from YAC 24-3-G, were restriction mapped with *Sal*I, *Not*I, *Asc*I, and *Sfi*I. The restriction map of YAC 15-6-H was consistent with the restriction maps of the genome in this region and of YAC 24-3-G (Fig. 2). However, the restriction map of YAC 5-12-E was not consistent with that of the *cenVII* region; since the terminal probe from YAC 24-3-G is somewhat repetitive, this clone is most probably from a different location in the genome. The 4.8-kb *Pac*I-*Eco*RI terminal fragment from the *TRP1* arm of YAC 15-6-H (Fig. 2) was subcloned into *E. coli*. This fragment hybridized weakly to several restriction fragments on *N. crassa* genomic Southern blots and strongly to 152-kb *Asc*I, 312-kb *Sfi*I, and 412-kb *Not*I restriction fragments (Fig. 3C, lanes a, s, and n, respectively), consistent with the physical location of YAC 15-6-H as shown in Fig. 2.

To complete the walk, the YAC library was probed with the *met-7*-distal terminal fragment from the insert of YAC 15-6-H, and four YAC clones hybridized strongly to the probe. These four YAC clones were restriction mapped with *Sal*I, *Not*I, *Asc*I, and *Sfi*I and ordered into a 205-kb contig. The restriction map of this contig was consistent with the restriction maps of the genome and overlapped with YAC clones 12-10-H, 15-6-H, and 24-3-G. A representative clone (19-5-B) from this contig extending the farthest in the *qa-2*$^+$ direction is diagrammed in Fig. 2. The overlap between YAC clones 12-10-H and 19-5-B was verified by probing Southern blots of *Eco*RI-digested AB1380/YAC 12-10-H, AB1380/19-5-B, AB1380/15-6-H, and AB1380/24-3-G with the *qa-2*$^+$-distal 8.2-kb fragment from the insert of YAC clone 12-10-H. This fragment, which had been recovered by rescue in *E. coli* and restriction mapped (see text below and Fig. 6), hybridized to the same set of five *Eco*RI restriction fragments on YAC clones 12-10-H and 19-5-B (Fig. 4). These five fragments are identical in size to the *Eco*RI fragments present within or adjacent to the 6-kb hybridization probe (see Fig. 6D), thus confirming the overlap and completion of the chromosome walk. A minimal set of overlapping YAC clones spanning the region between *qa-2*$^+$ and *met-7*$^+$ is shown in Fig. 2A. The contig is 750 kb long (19% of the length of lgVII).

Localization of centromeric DNA within the 750-kb YAC contig. Centromeric DNA within the 750-kb contig was localized by assaying the YAC clones shown in Fig. 2 for three properties common to centromeric DNA. These properties are high A+T content, greatly decreased meiotic recombination frequency, and the presence of centromere-specific DNA sequence repeats (11, 12, 28, 42).

The A+T content of the region was estimated by mapping the frequency of *Pac*I sites occurring across the YAC contig. The highest frequency of *Pac*I cutting was found in the 450-kb region between *qa-2*$^+$ and *met-7*$^+$ (Fig. 2C). A total of 73 distinct *Pac*I sites and clusters of sites were found within this region. (Clusters of *Pac*I sites which occurred within 1 to 2 kb of each other could not be resolved in this analysis.) However, no *Pac*I sites were seen outside the boundaries of this 450-kb *Pac*I "island." The A+T content of the DNA in this region is at least 67%, determined by the frequency of *Pac*I cutting, counting clusters of *Pac*I sites as single sites. This is 20% higher than the average A+T content (47%) of the *N. crassa* genome (29). To verify that the high frequency of *Pac*I cutting was due to a high A+T content and not due to an increase in nonrandomly distributed *Pac*I recognition sites, 1 kb of DNA from the A+T-rich end of YAC 12-10-H was sequenced (530 bp is shown in Fig. 8). This 1-kb segment of DNA has an A+T content of 64%, consistent with that estimated for the region by the *Pac*I analysis.

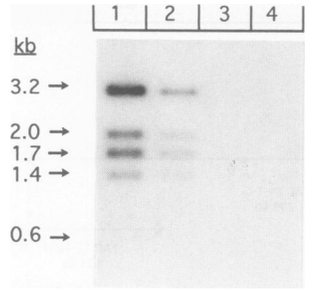

FIG. 4. Verification of overlap between YAC clones 12-10-H and 19-5-B. Genomic Southern gel blot of *Eco*RI-digested DNA from yeast strains AB1380/YAC 12-10-H (lane 1), AB1380/YAC 19-5-B (lane 2), AB1380/YAC 15-6-H (lane 3), and AB1380/YAC 24-3-G (lane 4) probed with labelled 8.2-kb *Cla*I-*Eco*RI terminal insert fragment from the *URA3*-proximal end of YAC 12-10-H. Fragment lengths are indicated (arrows).

Recombination frequencies within the genomic interval spanned by the YAC contig were determined by measuring the ratio of physical to genetic distances in previously characterized genetic intervals (Table 1). The recombination frequency in the 450-kb interval between *qa-2*$^+$ and *met-7*$^+$ is only 2.7 to 3.8% of that in the flanking regions (Table 1) and 0.2% of the estimated average recombination frequency over the entire genome (32, 35). The ratio of physical to genetic distance in the *qa-2-met-7* region is increased to 22,000 kb/cM.

Quantitative Southern blot analysis of repetitive sequences. Hybridization probes used for chromosome walking within the 450-kb region between *qa-2*$^+$ and *met-7*$^+$ contained repetitive sequences (Fig. 3). Repetitive sequences that hybridized to the 800-bp terminal fragment of YAC 12-10-H were further analyzed. In genomic Southern blots of *Eco*RI, *Hin*dIII, and *Sal*I digests, the 800-bp terminal fragment

TABLE 1. Recombination interference in the *cenVII* region

Genetic interval	Physical distance (kb)	Map units (cM)	Physical distance/genetic distance
qa-1 to *qa-2*	17	0.02[a]	850
qa-2 to *met-7*	450	0.02[a]	2.2×10^4
met-7 to *met-9*	6	0.01[b]	600

[a] These genetic intervals are from reference 10.
[b] This genetic interval is from reference 31.

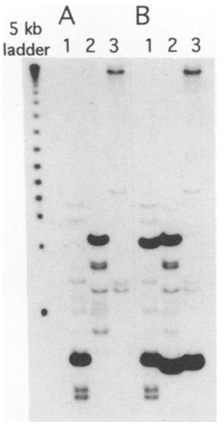

FIG. 5. Quantitative Southern blot hybridization of the repetitive 800-bp *Eco*RI-*Xho*I terminal fragment from YAC 12-10-H to *N. crassa* genomic DNA. (A) *N. crassa* genomic DNA cut with *Eco*RI (lane 1), *Hin*dIII (lane 2), and *Sal*I (lane 3) hybridized with the 800-bp terminal fragment from YAC 12-10-H. (B) The same digests as in A, hybridized with both the 800-bp terminal fragment and a 900-bp single-copy fragment from the *qa-2* gene. Both probes were hybridized simultaneously to the filter at equivalent specific activities and concentrations (see Materials and Methods).

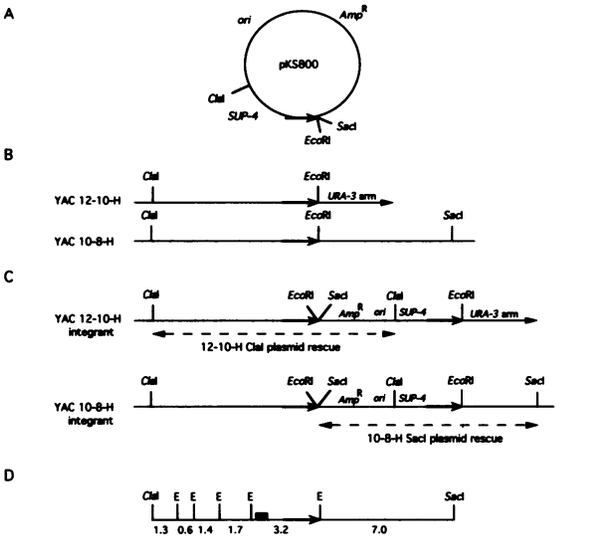

FIG. 6. Diagram of the vector and strategy used to rescue additional DNA flanking the 800-bp *Xho*I-*Eco*RI terminal fragment of YAC 12-10-H. (A) Yeast integration vector pKS800. The 800-bp *Xho*I-*Eco*RI terminal fragment of the insert of YAC 12-10-H is depicted on this plasmid and in the remaining panels (dark arrow). The 800-bp fragment contains a unique *Bgl*II site, which was cleaved to direct the homologous integration of the plasmid into the YAC clones diagrammed in B and C. (B and C) The *met-7*-proximal terminus of the insert in YAC 12-10-H and an overlapping portion of YAC 10-8-H before (B) and after (C) integration of pKS800. Regions flanking the 800-bp fragment that were cloned into *E. coli* by plasmid rescue are indicated (broken line). (D) Composite restriction map of the DNA rescued into *E. coli* from YAC clones 12-10-H and 10-8-H. E, *Eco*RI. Subclones of the rescued DNA, which were used for analysis of repeat structure, are indicated by their size (in kilobases). The 530-bp *Sau*3AI fragment used for comparisons of centromeric DNA sequence repeats (hatched box) is shown.

hybridizes strongly to one genomic band and weakly to >20 bands (Fig. 5A).

The genomic copy numbers of the restriction fragments that hybridized to the 800-bp fragment were determined by quantitative Southern hybridization blots (Fig. 5). *Eco*RI, *Hin*dIII, and *Sal*I genomic Southern blots were hybridized simultaneously with the 800-bp terminal fragment of YAC clone 12-10-H and a single-copy 900-bp *Bgl*II-*Hin*dIII fragment from within the coding region of the *qa-2*$^+$ gene. The 3-kb *Eco*RI and 13-kb *Hin*dIII bands that hybridized strongest to the 800-bp YAC terminal fragment gave hybridization signals of nearly equal intensity to those of the 12-kb *Eco*RI and 2.8-kb *Hin*dIII fragments hybridizing to the single-copy 900-bp hybridization probe. The restriction fragments that hybridize strongly to the 800-bp terminal fragment from YAC 12-10-H are, therefore, present in the genome in approximately a single copy. The restriction fragments that hybridize weakly to the 800-bp terminal fragment must, therefore, contain only partial homology to the hybridization probe.

Isolation of additional repetitive DNA from the *cenVII* region. *N. crassa* DNA surrounding the repetitive 800-bp *Xho*I-*Eco*RI terminal fragment of insert DNA in YAC 12-10-H was subcloned from YAC clones 12-10-H and 10-8-H (a YAC that overlaps with 12-10-H [Fig. 6B]) into *E. coli*. The vector and strategy utilized to obtain 15 kb of DNA flanking the 800-bp terminal fragment is shown in Fig. 6. Briefly, the 800-bp fragment was cloned into an *E. coli* plasmid vector (pBluescript) that contained the *S. cerevisiae SUP4* gene (see Materials and Methods). This construct, named pKS800 (Fig. 6A), was cleaved at a unique *Bgl*II restriction site within the 800-bp sequence, and the linearized DNA was used to transform yeast strains AB1380/12-10-H and AB1380/10-8-H, which harbor a *SUP4* suppressible *ade2* ochre mutation. DNA was prepared from Ade$^+$ transformants in which pKS800 had integrated into a homologous region within the YAC insert. The transformant DNA was cleaved with a restriction enzyme that would excise the *E. coli* vector along with flanking DNA from the YAC inserts, the resulting fragments were circularized by treating DNA with ligase at high dilution, and finally the circularized fragments were used to transform *E. coli*, selecting for ampicillin resistance. Approximately 15 kb of DNA surrounding the 800-bp region was isolated in two rescue plasmids (Fig. 6C). This 15 kb of DNA was further subcloned into the six DNA contiguous fragments shown in Fig. 6D. Each of these six fragments, when separately hybridized to genomic Southern blots of *N. crassa* DNA cut with *Eco*RI, *Hin*dIII, and *Sal*I, revealed the presence of repetitive sequences (data not shown). To assay for internal homology within this 15-kb repetitive region, the six restriction fragments shown in Fig. 6B were fractionated by gel electrophoresis and blots were probed separately with each labelled fragment. No homology between these fragments was observed (data not shown), indicating that the repetitive sequences are relatively dispersed and are not tandemly arranged.

RFLP mapping of repetitive sequences. The genomic locations of 20 restriction fragments with homology to repetitive DNA from the *cenVII* region were mapped by RFLP analysis. DNAs from the multicent-2-a strain (a derivative of the wild-type Oak Ridge strain) and of the wild-type Mauriceville-1c-A strain (27) of *N. crassa* were digested with the restriction enzymes *Pvu*II, *Bam*HI, *Sma*I, *Pac*I, *Kpn*I, *Cla*I, *Hin*dIII, and *Sal*I, and Southern blots of the digests were

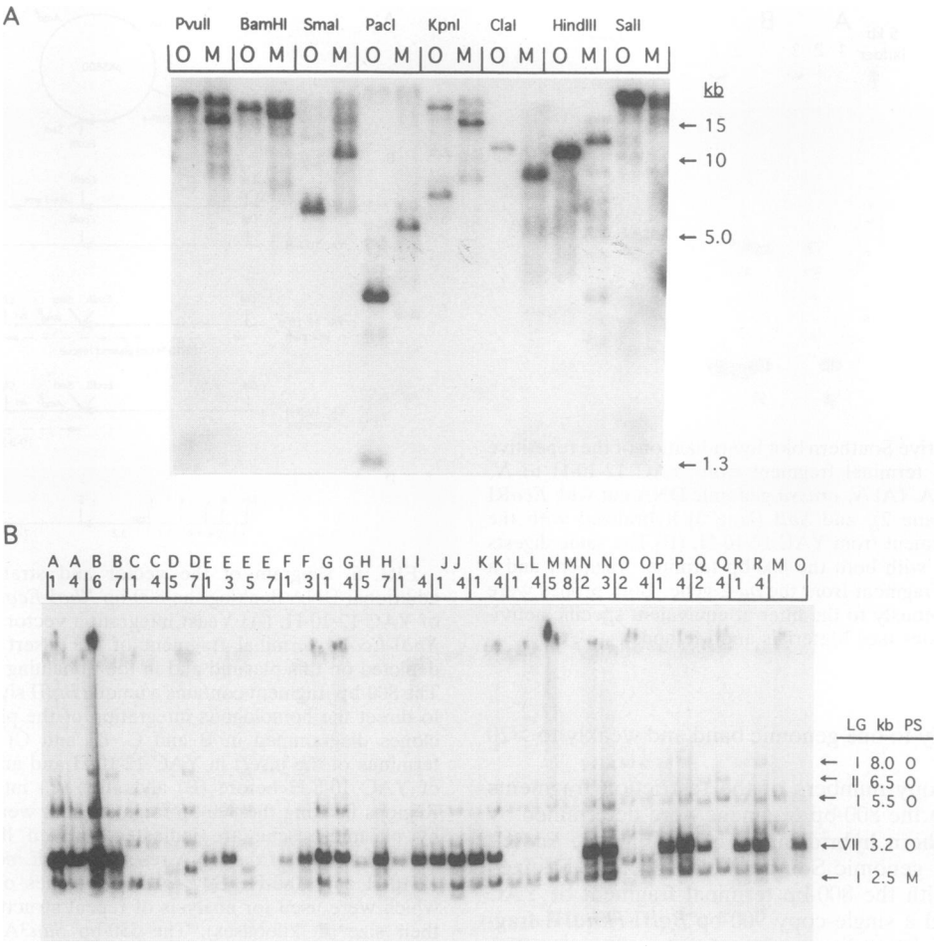

FIG. 7. RFLP analysis using a repetitive probe from the *cenVII* region. (A) Characterization of useful RFLPs that hybridize to the repetitive terminal fragments from the insert of YAC 12-10-H. A Southern blot of genomic DNAs from a derivative of the wild-type Oak Ridge (O) strain and the Mauriceville (M) strains cleaved with the restriction enzymes *Pvu*II, *Bam*HI, *Sma*I, *Pac*I, *Kpn*I, *Cla*I, *Hin*dIII, and *Sal*I and probed with the repetitive 3.2-kb terminal *Eco*RI fragment from the *URA3*-proximal end of YAC 12-10-H is shown. Fragment lengths (at arrows) are shown. (B) Sample autoradiograph from the RFLP analysis. Genomic DNAs isolated from 38 segregants from 18 ordered asci were cleaved with the restriction enzyme *Pac*I, fractionated by agarose gel electrophoresis, blotted to a membrane, and probed with the 800-bp *Xho*I-*Eco*RI terminal fragment from the insert of YAC 12-10-H. Two exposures (see Materials and Methods) were required for complete analysis of the segregation patterns of these RFLPs. The lighter exposure is shown and, therefore, some of the bands may appear weak. The 18 asci, lettered A through R above, each contained eight spores in a linear array. The 38 segregant spores chosen for further analysis are numbered 1 through 8 beneath the asci from which they were derived, with respect to their original position within the ascus. Segregants numbered 1 through 4 and 5 through 8 are derived from sister spores. Individual spores in a given ascus in each of the pairs of spores, 1 and 2, 3 and 4, 5 and 6, and 7 and 8, are derived from the same progenitor cell which has undergone a postmeiotic mitosis and are, therefore, clones of each other. Representatives from all four clonal pairs of spores and from all four sister spore pairs are shown for ascus E. RFLPs that could be unambiguously scored from the two exposures of this Southern blot are indicated with arrows. The linkage group (LG), fragment length, and parental strain (PS) that carries the positive hybridization signal of a given RFLP are indicated.

probed with the repetitive 800-bp *Xho*I-*Eco*RI and 3.2-kb *Eco*RI terminal fragments from YAC 12-10-H. Polymorphic bands between the two strains were seen in all restriction digests (Fig. 7A).

DNAs obtained from a set of 38 segregants from ordered asci derived from a cross of the above strains were digested with *Pac*I and *Kpn*I, and Southern blots of the digests were hybridized with the 800-bp and 3.2-kb repetitive probes. Parental alleles from 19 restriction fragments which hybridized to the above probes segregated predominantly to sister spores (Table 2 and Fig. 7B). All 19 restriction fragments are therefore centromere linked. Comparison of the segregation data derived from the centromere-linked restriction fragments with those of previously mapped markers localized the 19 restriction fragments to centromeres in all seven linkage groups of *N. crassa* (Table 2). One of the twenty restriction fragments was located on the right arm of lgV, near the centromere region (Table 2). Additionally, one of the restriction fragments that hybridized to the repetitive probe, and mapped to the centromere region of lgVII, seemingly segregated to nonsister spores in one ascus (Fig. 7B, ascus Q). Interestingly, spore 2 of this ascus always contained a strongly hybridizing band that mapped to this location, independent of the hybridization pattern for that band in the parental strains, as if both parental alleles were present for this location in this spore (data not shown).

Partial sequence analysis of three copies of the centromere-linked repetitive sequence. To determine the nature of the

TABLE 2. RFLP analysis indicating map locations of repetitive DNA sequences hybridizing to probes from the *cenVII* region

Linkage group	Probe length[a]	Digest	Characteristics		Map location[c]
			RFLP band size (kb)	Parental strain[b] with RFLP band	
I	3.2 kb	*Pac*I	9.5	M	Cen
	3.2 kb	*Pac*I	2.8	M	Cen
	800 bp	*Pac*I	8.0	O	Cen
	800 bp	*Pac*I	5.5	O	Cen
	800 bp	*Pac*I	2.5	M	Cen
	800 bp	*Kpn*I	37	M	Cen
II	800 bp	*Pac*I	6.5	O	Cen
III	800 bp	*Kpn*I	32	O	Cen
	800 bp	*Kpn*I	22	M	Cen
IV	800 bp	*Pac*I	3.0	O	Cen
V	3.2 kb	*Pac*I	1.6	O	Cen
	800 bp	*Kpn*I	5.5	O	R. arm
VI	3.2 kb	*Kpn*I	2.7	M	Cen
VII	3.2 kb	*Pac*I	4.8[d]	M	Cen
	3.2 kb	*Pac*I	3.2[d]	O	Cen
	3.2 kb	*Kpn*I	25[d]	O	Cen
	3.2 kb	*Kpn*I	8.0[d]	O	Cen
	800 bp	*Kpn*I	8.0[d]	O	Cen
	800 bp	*Kpn*I	17[d]	M	Cen
	800 bp	*Kpn*I	11	O	Cen

[a] The 3.2-kb *Eco*RI terminal fragment and the 800-bp *Xho*I-*Eco*RI terminal fragment of the insert of YAC 12-10-H, used as hybridization probes, were cloned from the YAC into *E. coli* as described in the text and diagrammed in Fig. 6.

[b] O, Oak Ridge-derived multicent-2-a (27); M, Mauriceville-1c-A (27).

[c] Cen, centromere; R. arm, right arm.

[d] These restriction fragments hybridize relatively strongly to the hybridization probes used in this analysis. As for the quantitative Southern blot, bands from which the probes were derived had the highest signal intensities.

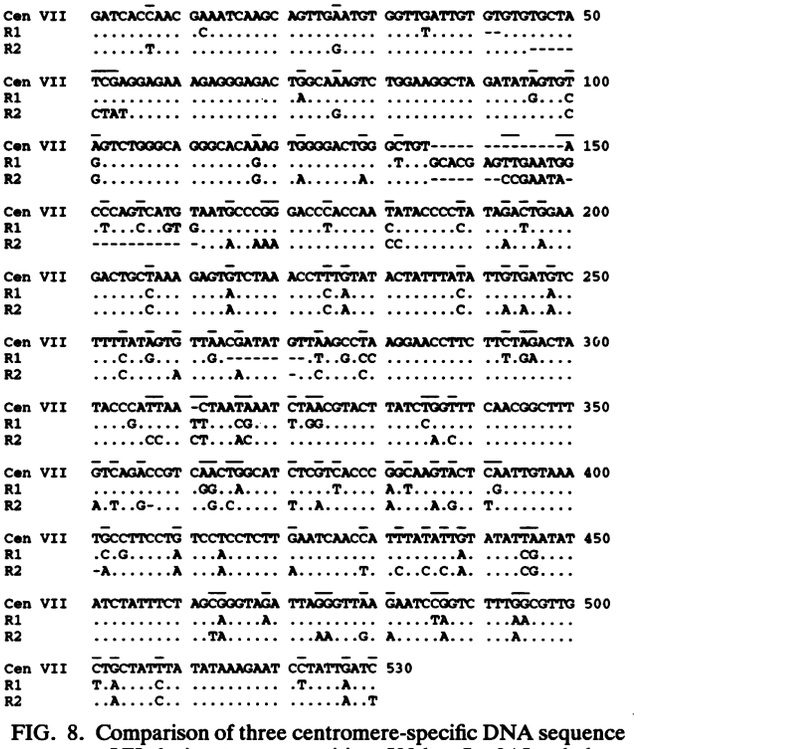

FIG. 8. Comparison of three centromere-specific DNA sequence repeats. *cenVII* designates a repetitive 530-bp *Sau*3AI subclone derived from the A+T-rich region of YAC 12-10-H (Fig. 6). R1 and R2 are subclones derived from two independently isolated restriction fragments that hybridized to the *Sau*3AI fragment. Identity between sequences (.) and probable positions of insertion or deletion (–) are indicated. A bar above a base indicates a transitional difference between two or more repeats at that position.

sequence divergence among the centromere-linked repetitive sequence fragments, a plasmid library of *Eco*RI-digested *N. crassa* genomic DNA was prepared and 20 clones that hybridized to the 7.9-kb *Cla*I-*Eco*RI terminal fragment of YAC 12-10-H (Fig. 6) were isolated. The 20 repetitive-sequence clones were probed with a 530-bp *Sau*3AI fragment from within the YAC 12-10-H rescue plasmid (Fig. 6). Two clones that hybridized strongly to the *Sau*3AI fragment were identified and named R1 and R2. These DNAs were digested with *Eco*RI and *Sau*3AI, and small fragments that hybridized to the 530-bp probe were subcloned and sequenced. Comparisons of the sequences of the subclones from R1 and R2 and that of the 530-bp fragment from within the 450-kb region are shown in Fig. 8. The three repeats were highly homologous over the entire length of the clones, with sequence similarities ranging from 84 to 87% among repeats. They were A+T rich, with A+T contents of 59, 61, and 59% for the *cenVII* fragment, R1, and R2, respectively (Fig. 8). Base differences among the three repeats involved predominantly transitional changes (86%).

The genomic location of the repetitive DNA sequences R1 and R2 could not be determined. These fragments hybridized to multiple genomic bands; however, unlike the DNA fragments obtained from the terminus of YAC 12-10-H, these DNA fragments did not hybridize preferentially to a single band that corresponded to the restriction fragment from which they were derived. RFLP analysis using these repetitive probes was done, but there was no way to distinguish which of the multiple bands hybridized to these DNA fragments corresponded to the genomic location of the probe (data not shown).

DISCUSSION

Centromeric DNA from many organisms can be characterized as recombination-deficient islands of A+T-rich DNA containing centromere-specific repeated sequences. A 750-kb chromosome walk, 19% of the total chromosome length, on lgVII of *N. crassa* was completed. The walk encompasses the centromere-linked *qa* gene cluster and *met-7*. A 450-kb region flanked by the *qa* gene cluster and *met-7* within this 750-kb contig has properties characteristic of centromeric DNA. Recombination in the 450-kb region is only 0.2% of the average recombination frequency of the genome (32, 35), the A+T content is 20% higher than the average A+T content of the genome (29), and repetitive sequences from within this region hybridize to a family of repeats which are present at or near centromeres on all seven chromosomes of *N. crassa*. This 450-kb region is flanked on both sides by DNAs with higher recombination frequencies and lower A+T contents, strongly suggesting that the entire *cenVII* locus is contained within this region. Since the position of the *qa* gene cluster relative to the centromere has not been established, however, there is no

definitive genetic evidence that the centromere is contained within the 750-kb contig. Definitive evidence for the presence of a functional centromere within this region might eventually be obtained by introducing the YACs containing these cloned DNA segments into *N. crassa*. As in *S. cerevisiae* and *S. pombe*, clones that contain an *N. crassa* centromere may function as artificial chromosomes, replicating autonomously and faithfully segregating during both mitotic and meiotic cell divisions. However, the DNA required in *cis* to specify a functional centromere in *N. crassa* may be too large to be contained on any one of the four YAC clones that span the 450-kb region; it might be necessary to fuse neighboring YACs within this region and assay the resulting clones for centromere function.

Reduction of recombination frequencies within genomic regions in close proximity to centromeres is known as the centromere effect. The centromere effect has been noted in many eukaryotes, but no evidence of this effect in *N. crassa* has been reported (11). Our analysis has shown that recombination frequencies do decrease dramatically near *cenVII*; recombination is nearly silent in the 450-kb region between the *qa* gene cluster and *met-7*, a region spanning 11% of the total length of the chromosome. The centromere effect in *N. crassa* is much greater than that seen in either *S. pombe* (17) or *S. cerevisiae* (20), and as in higher eukaryotes, it extends into the arm of the chromosome, diminishing gradually in the 200-kb interval from *cenVII* to *wc-1* (22). Centromeric chromatin in *N. crassa* may, therefore, be similar to that seen in more complex eukaryotes, where high levels of recombination interference in centromere-proximal regions have been observed (11).

Although the 450-kb *cenVII* region shares most of the general properties of centromeric DNA, the DNA sequence repeats appear to be unusual. Repeats in higher organisms at the centromere are highly repetitious, containing many copies of a relatively short repeat sequence. In *S. pombe*, fairly long sequence repeats (~6 kb), in relatively few copies, are present at the centromeres, with DNA sequences highly conserved among copies of these repeats. *S. cerevisiae* centromere regions contain no sequence repeats. The *N. crassa* centromeric sequence repeats characterized in this work, like those of *S. pombe*, are relatively long and do not contain short satellite repeat sequences; however, unlike *S. pombe* these repeats are highly divergent. In addition, no evidence for large inverted repeat sequences, as seen in *S. pombe*, was found in either the physical map of the 750-kb contig or in Southern hybridizations of repetitive probes from this region.

The high sequence divergence, the high A+T content, and the predominantly transitional differences observed between *N. crassa* centromere-specific sequence repeats strongly suggest operation of the repeat-induced point mutation (RIP) system. RIP is a process of mutation in *N. crassa* in which C-to-T transitions occur with high frequency within repeated sequences (39, 40). RIP has a nearest-neighbor bias, with C-to-T transitions occurring in the dinucleotides dCpdA > dCpdT > dCpdG > dCpdC; and for trinucleotides, RIP occurs most frequently within the sequence dCpdApdT. The centromere repeat sequences in Fig. 8 show the same nonrandom preferences of dinucleotides in which putative C-to-T transitions have occurred among repeats, with dCpdA > dCpdT > dCpdG > dCpdC occurring at 46, 24, 17, and 12%, respectively. Furthermore, these sequence repeats contain a low frequency of the dCpdApdT trinucleotide (0.8%) and a high frequency of the dTpdApdT trinucleotide (4.4%), the RIP product of dCpdApdT, as would be expected for sequences that have undergone RIP.

If RIP is occurring in the centromere repeats of *N. crassa* and if these repeats are required for centromere function, then a powerful in vivo mutagenesis experiment has been ongoing for millennia. If an in vivo functional assay for centromeric DNA can be developed for *N. crassa* and a minimal centromere structure elucidated, a comparison of the highly divergent functional centromere repeat sequences should quickly pinpoint conserved sequences required for function and protein binding.

In addition to providing the groundwork for a potentially exciting centromere model, this study also represents a solid foundation for a genome project on *N. crassa*. By walking in a YAC library, we have been able to clone the longest continuous segment of *N. crassa* DNA isolated to date, spanning 750 kb (19% of lgVII's length) in only four steps. The entire 750-kb region is contained on a set of four overlapping YAC clones; this translates into 187 kb of coverage per clone. The entire 42.9-Mb genome of *N. crassa* could therefore be isolated on an estimated 230 YAC clones. *N. crassa* is a classic experimental organism with approximately 700 genetically mapped loci (33). The detailed genetic map, in conjunction with a complete physical map and overlapping clone collection, would make *N. crassa* an ideal system in which to study eukaryotic genome organization and gene function.

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