A Schizosaccharomyces pombe artificial chromosome large DNA cloning system

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ABSTRACT

The feasibility of using the fission yeast, Schizosaccharomyces pombe, as a host for the propagation of cloned large fragments of human DNA has been investigated. Two acentric vector arms were utilized; these carry autonomously replicating sequences (ars elements), selectable markers (ura4* or LEU2) and 250 bp of S.pombe terminal telomeric repeats. All cloning was performed between the unique sites in both vector arms for the restriction endonuclease NotI. Initially the system was tested by converting six previously characterized cosmids from human chromosome 11p13 into a form that could be propagated in S.pombe protoplasts in the presence of lipofectin. Prototrophic ura + leu + transformants were obtained which upon examination by PFGE were found to contain additional linear chromosomes migrating at lengths between 100 and 500 kb with a copy number of 5–10 copies/cell. Hybridization analyses revealed that these additional bands contained human DNA. Fluorescent in situ hybridization (FISH) analyses of several independent clones indicated that the inserts were derived from single loci within the human genome. These analyses clearly demonstrate that it is possible to clone large fragments of heterologous DNA in fission yeast using this S.pombe artificial chromosome system which we have called SPARC. This vector–host system will complement the various other systems for cloning large DNA fragments.

INTRODUCTION

Yeast artificial chromosomes (YAC) replicate once per cell cycle, are maintained as linear episesomes and segregate equally to both daughter cells during mitosis (1,2). YACs were first developed for studying chromosome structure and function in the budding yeast, Saccharomyces cerevisiae (1). Subsequently YACs were exploited for cloning large fragments of DNA from heterologous organisms to facilitate the effort to physically map large genomes (2). The original YAC clones were relatively small with an average insert size of ∼250 kb. The development of more sophisticated methods for size selecting the target DNA molecules and minimizing shearing forces by including polyamionic agents such as spermidine to artificially condense the DNA allows human fragments of up to 1500 kb to be cloned in S.cerevisiae YACs (3–6).

The low copy number of YACs has made them hard to purify in reasonable quantities for further manipulations. To overcome this, methods have been developed where YACs are amplified by conditional inactivation of the centromere and inclusion of an amplifiable selectable marker (7,8). However, such amplification requires a retrofitting step to be performed on each YAC of interest and not all YACs amplify efficiently. The purification of YACs is also hampered by the fact that the 17 endogenous chromosomes of S.cerevisiae (150–2500 kb) frequently co-migrate with a YAC making it difficult to purify away from yeast genomic DNA. Certain sequences, tandem repetitive arrays in particular, frequently suffer deletions when cloned in the YAC system (9). Deletion of the S.cerevisiae RAD52 gene facilitates the propagation of long tandem arrays of human centromeric tandem alphoid repeats (9). It has also been found that a high proportion of YACs in some human libraries are chimeric, making their analyses more difficult and their usefulness for chromosome walking limited (10).

Other large DNA cloning systems have been developed making use of the bacterial phage P1, which allows heterologous DNA of up to 100 kb to be packaged and propagated upon infection of Escherichia coli (11). Manipulation of the E.coli episome which carries the F factor resulted in the bacterial artificial chromosome (BAC) cloning system which propagates human DNA inserts of up to 700 kb (12–15). Melding of the BAC and P1 systems has resulted in the development of PACs, which no longer require packaging into phage and are replicated to a relatively low copy number in E.coli, facilitating the purification of large DNA (16,17).

The fission yeast, Schizosaccharomyces pombe, has a similar sized genome (13.8 Mb) to that of S.cerevisiae but it is packaged into only three chromosomes of 5.6, 4.7 and 3.5 Mb (18). Thus, additional minichromosomes of <3000 Mb can be easily separated and gel purified from host chromosomes on pulsed field gels. Previously, we have shown that protoplasts of the fission yeast can be transformed at a high frequency by inclusion of the

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cationic liposome, Lipofectin (19,20). This transformation protocol was also shown to allow DNA molecules of 500 and up to 750 kb derived from minichromosomes to be transformed intact from molten agarose gel slices into *S. pombe* (19,20).

Cis-acting DNA elements with replication origin function have been isolated and analysed in fission yeast (21,22). The terminal telomeric repeats have been cloned and characterized (23,24). A large region of at least 15 kb is required to provide reasonable centromere function in *S. pombe* (25,26; reviewed in 27). Since these centromeric regions are difficult to propagate in *E. coli* this precludes their incorporation into a vector system (25,26). Previously, we have shown that linearized plasmids containing the *ura4* or *LEU2* marker, a replication origin contained within an autonomously replicating sequence (ars) and *S. pombe* chromosomal terminal telomeric repeats attached at each end can be propagated as linear episomes upon transformation into *S. pombe* cells (24). Here we examine the feasibility of using these *ura4* and *LEU2* plasmids as arms between which heterologous (human) DNA can be cloned.

**MATERIALS AND METHODS**

**Plasmids and cosmids**

pEN51 and pEN53 were described previously (24).

The cosmids from human chromosome 11p13 were provided by W.Bickmore and were described previously (28). Cosmids I and II are lawrist4 based and are H11148 and J44 (28). Cosmids III–VI are pWE15 based and are U22, U143, U447 and U833, respectively (29).

**Culture media**

Standard media were used to grow *S. pombe* (30) and *E. coli* (31). For protoplast preparation *S. pombe* was grown in EMMG (3.76 g/l glutamic acid) with 0.5% glucose.

**Schizosaccharomyces pombe strains used**

SP813 (FY367); *h*+ *ade6-210 leu1-32 ura4-D18* (19).

FY562: *h*+ *leu1-32 ura4-D18 rec55-36* (derivative of BG48; 32).

**DNA preparation and analyses**

Standard methods were used to prepare normal sized *S. pombe* DNA (30). DNA was digested under conditions recommended by suppliers. For conventional agarose gel electrophoresis DNA was separated on 0.6–1.2% agarose gels using standard horizontal rigs. Low melting point agarose at 1% was used for preparation of DNA fragments for use as probes, etc. All gels were run in 0.5× TAE. Pulse field gel electrophoresis (PFGE) was performed on a Bio-Rad CHEF-DR11 rig. Routine analytical gels were 1% agarose in 0.5× TBE DNA, preparative gels were 1% low melting point agarose in 0.5× TAE. A range of running conditions were used to separate different sized molecules.

DNA was transferred from agarose gels (conventional and PFGE) to GeneScreen membrane filters (DuPont) and hybridized with labelled probes by standard procedures (31). Signal from hybridized filters was visualized by exposure to a Molecular Dynamics PhosphorImager screen.

**Preparation and digestion of large *S. pombe* DNA in agarose plugs**

Agarose plugs containing chromosome sized *S. pombe* DNA were prepared as previously described (20). For digestion with restriction endonucleases plugs were washed at least four times in TE (10 mM Tris–HCl, 1 mM EDTA, pH 8) including an overnight wash and then washed in 1× restriction endonuclease buffer (as recommended by manufacturer) three times for 30 min. Plugs were then covered by buffer containing the appropriate restriction enzyme (∼30 U/µg DNA) and incubated overnight.

**Schizosaccharomyces pombe protoplast transformation**

Protoplasts were prepared and transformation performed with large DNA molecules using Lipofectin (Gibco BRL) as previously detailed (19,20). Modifications were as follows: cells were grown in EMMG + 0.5% glucose to 1.0–2.0 × 10⁷ cells/ml and a higher density of cells (0.5–1.0 × 10⁸ cells/ml) was used during digestion with NovoZym 234 in SP2 (20) at 5–10 mg/ml. These changes consistently gave a higher proportion of intact protoplasts within 30 min of digestion.

**Catch-linker PCR and FISH**

SPARC DNA was labelled with biotin-11-dUTP by nick translation after isolation from PFGE gels and ligation to catch-link oligonucleotides (33).

Metaphase spreads of human chromosomes were prepared, hybridized and signal detected as previously described (34). Mounted slides were viewed on a Zeiss Axioplan microscope. The images were captured with a Photometrics CCD camera and processed using IPLab spectrum software with Digital Scientific extensions.

**RESULTS**

**Strategy for cloning DNA as linear episomes in *S. pombe***

The plasmids pEN51 and pEN53, used as the vector arms for cloning linear DNA in *S. pombe*, were described previously (24). The strategy adopted for cloning *NotI* fragments in *S. pombe* is outlined in Figure 1. Briefly, digestion of pEN51 (*ura4*) or pEN53 (*LEU2*) with SacI exposes the telomeric repeats for the *de novo* seeding of telomeres in transformants. Treatment with alkaline phosphatase suppresses the formation of SacI–SacI multimers during ligation. After phosphatase treatment, digestion of each plasmid with *NotI* releases the cloning site and the 6.6 and 7.7 kb pEN51 and pEN53 vector arms are gel purified away from the small 1.1 kb telomere fragment. Following digestion, DNA from test cosmids or total human genomic DNA was ligated with pEN51 and pEN53 arms prepared as described above. After ligation to *NotI*-cut genomic DNA the products in the desired size range were gel concentrated, purified and transformed into the host strains of *S. pombe*, SP813(FY367) or FY562.

**Propagation of human cosmids as linear episomes in *S. pombe***

Six previously characterized cosmids containing human DNA mapping to chromosome 11p13 were initially chosen to test the ability of the SPARC cloning system to maintain and propagate human DNA (28,29). Two of the cosmids (I and II) utilized were originally isolated from a library made in the lawrist4 cosmid vector (28). These two cosmids contain only a single site for the
In transformations with the six cosmid–SPARC arm ligations ~50 transformants were obtained with between 50 and 100 ng of cosmid–SPARC arm ligation products. Undigested high molecular weight DNA from 25 ura+ leu+ transformants resulting from transformation with the Cos1-SPARC ligation was examined by PFGE and staining with ethidium bromide (12 are shown in Fig. 2A). Twenty-three of these transformants contained a band of the expected size of 60 kb. One contained a band of 120 kb, perhaps representing a dimer. In the remaining transformant no linear episome was apparent.

Southern hybridization analyses using total human DNA as a probe revealed that 24 out of these 25 SPARCs contained human DNA (Fig. 2B). In addition to the expected 60 kb band all positive transformants contained a weaker 120 kb band and in some a band of 180 kb could be detected. Further analyses indicated that these larger bands resulted from multimerization of the SPARC through the vector arms and not rearrangement of the human cosmid DNA insert (below and Fig. 3). It is possible that multimerization of the SPARC has a selective advantage in that it may increase the stability of these acentric linear episomes. Consistent with this, no multimers were detected when larger fragments (>100 kb) of human DNA were cloned between the SPARC arms (below and Figs 4 and 7). Similar results were obtained with all six test cosmids; in all cases linear episomes of 60 kb were recovered at a high frequency.

The integrity of the human cosmid insert was next examined by releasing the insert from the SPARC by digestion with Not1 and comparison with the size of the Not1-digested starting cosmid. In the four Cos1 transformants examined in Figure 2C it is clear that all contain an insert of similar size to that released by Not1 digestion of the starting cosmid itself. To test more rigorously their integrity, cosmid-SPARC DNA, from several transformants, was digested with HindIII and probed with total human DNA to ‘fingerprint’ them with respect to fragments containing dispersed repetitive DNA elements. The fingerprints of all three Cos1-SPARC transformants examined in this way were identical to each other and to the original cosmid itself apart from the cosmid-SPARC Not1 junction fragment. Similar extensive analyses was performed on the Not1 fragments from the four pWE15 cosmids (CosIII–IV) cloned in SPARCs. Most ura+ leu+ transformants examined contained an undigested monomeric linear episome of the expected size, 45–55 kb (Fig. 3A–D). Digestion with Not1 of most transformants examined revealed a SPARC insert of size equivalent to that released from the parental cosmid. Fingerprinting by digestion with the frequently cutting enzymes EcoRI or HindIII in addition to Not1 demonstrated that the structure of the human DNA propagated in these linear SPARCs in S. pombe was identical to the parental cosmid DNA (Fig. 3E and F; data not shown). Thus the human 11p13 DNA from cosmids I–VI can be propagated intact in S. pombe on these acentric SPARC episomes. These initial experiments suggested that the SPARC system might allow a larger fragment of human DNA to be cloned and propagated in fission yeast.

### Cloning large Not1 fragments of human DNA in S. pombe SPARCs

The apparent lack of rearrangement of the six human cosmid DNA inserts when propagated in S. pombe as SPARCs was encouraging. Next we investigated whether fragments of human DNA of >100 kb could be cloned directly from a Not1 digest of
were digested with human DNA probe are identical in size after propagation as SP ARCs in as in (B). The phosphorimage indicates that all dIII and a SP ARC–CosI to the original CosI cosmid DNA. The largest band in T1, T2 and T3 represents prepared from the untransformed host.

The dimerization event is unknown. To further assess the integrity of these CosI-SP ARCs three of the above DNAs (60 kb) and in addition all contain a putative dimer of 120 kb. (∼)

Four of the CosI-SP ARC transformants from above (A and B) were chosen for further analyses. Undigested and Hin dIII or Hin dIII junction fragment. In (A–D) SP813 represents DNA from endogenous fission yeast chromosomes by PFGE on a low melting point agarose gel under conditions where DNA fragments of >200 kb co-migrated at the region of limiting mobility. Under these conditions unligated SPARC vector arms and smaller NotI fragments migrated well away from the target ligation products, which become concentrated at the region of limited mobility as reported previously (3,5). A large well of a PFGE gel was filled with plugs from the same ligation reaction. After separation the region of limiting mobility was identified by staining the outer edges of the lane with ethidium bromide and cutting out the relevant intervening region of the gel.

A gel slice containing the ligation products was then melted in TEN at 65°C and used to transform protoplasts of the strain FY562 in the presence of Lipofectin (19,20). This strain, FY562, contains the rec55-36 mutation which was originally selected as a hypo-recombination mutant that displayed a 10-fold lower frequency of recombination between two tandemly arranged copies of the ade6 gene (32). This strain was utilized to try and minimize rearrangement of tandemly repeated elements within the cloned human fragments. Twenty-five ura+ leu+ transformants were obtained from one transformation with this ligation mix. Undigested high molecular weight DNA was prepared and analysed by PFGE. All SPARCs could be easily visualized by staining these gels with ethidium bromide and were distinct from the endogenous chromosomes (Fig. 7). After blotting, hybridization of filters with labelled total genomic human DNA revealed the presence of additional bands in most of the transformants migrating between 80 and 450 kb (Fig. 4).

The 10 transformants with the largest human DNA inserts were chosen for further analyses. Hybridization of a Southern blot of a PFGE gel with labelled total human DNA is shown in Figure 4. SPARCs 1, 2, 10, 12, 14, 15, 20 and 21 contain one main band and one or more bands of weaker intensity. The origin of these additional molecules is unknown but they could result from rearrangement of the original main band SPARC and subsequently persist in the population at a low level. Alternatively, the Lipofectin-mediated transformation procedure might induce the uptake of multiple SPARC molecules by one cell. Restreaking of these transformants on selective plates for single colonies revealed that the different bands could be isolated as independent clones, suggesting that minor bands do not arise by continual rearrangement of the major SPARC band (data not shown).

**Mapping of SPARCs by FISH to human metaphase spreads**

To test if the DNA cloned in these SPARCs originated from a single site in the human genome the SPARC DNA was separated from endogenous fission yeast chromosomes by PFGE on a low molecular weight total human genomic DNA and propagated by the SPARC system. The strategy used was as outlined in Figure 1. High molecular weight total human DNA was prepared by standard methods (28) and digested with NotI in agarose plugs. Digested plugs were equilibrated by incubation in TEN (10 mM Tris–HCl, pH 8, 1 mM EDTA, 100 mM NaCl) at room temperature for 1 h and then melted at 65°C. The molten plug was cooled to 37°C and the prepared SPARC arms were mixed in and the agarose reformed as a 100 µl plug in an appropriate mould. The reformed plug was equilibrated for 1 h with an excess of 1× ligation buffer, after which ligase was added. To check that ligation had occurred a portion of a ligase-treated plug was examined by PFGE (not shown). Plugs which displayed a reasonable amount of ligation products were fractionated by PFGE on a low melting point agarose gel under conditions where DNA fragments of >200 kb migrated at the region of limiting mobility.

Undigested high molecular weight DNA was prepared and treated as in (B). The resulting phosphorimage clearly shows that 11 of the transformants shown contain a linear SPARC of the expected size for a CosI-SP ARC (∼)

Mapping of SPARCs by FISH to human metaphase spreads

![Figure 2](http://nar.oxfordjournals.org/baspa)
Figure 3. Analyses of CosIII–VI-SPARCs transformed and propagated in S. pombe. (A–D) As in Figure 2C, undigested and NotI-digested high molecular weight DNA prepared from two independent CosIII–VI SPARC transformants (T1 and T2) were separated by PFGE. These gels were Southern blotted and hybridized with [32P]CTP-labelled total genomic human DNA. The phosphorimages show that the human SPARC inserts are identical in size to the original NotI inserts of these human cosmids propagated in E. coli. (E and F) DNAs from two independent CosIII- and CosVI- SPARC transformants (T1 and T2) were digested with NotI and either HindIII or EcoRI and compared with the same digest of CosIII and CosVI DNA amplified in E. coli. Digests of CosIII and VI with HindIII or EcoRI alone are also included. After conventional electrophoresis the resulting gel was treated as in (A). The phosphorimages of these NotI and HindIII- or EcoRI-digested DNAs show the human DNA inserts were propagated intact as SPARCs in S. pombe and are identical to the original cosmid DNA insert. FY562 in (A–E) represents DNA prepared from the untransformed host S. pombe strain.

SPARC copy number and transformation efficiency

During the course of this work it appeared that these acentric, linear SPARC episomes might be propagated at greater than one copy per cell since even small SPARCs of 60–100 kb could be easily detected by staining gels with ethidium bromide (Figs 2A and 7). To determine the copy number in a population of cells where the SPARC is maintained under selective conditions (EMMG, no leucine or uracil), a labelled probe was employed which detects the ars1 fragment present on the pEN53 arm of the SPARCs and the single copy of ars1 residing in the middle of chromosome 1 close to rad4. Hybridization of this ars1 probe to Southern blots of DNA digested with ClaI allowed the SPARC-borne ars1 copy number to be quantified relative to genomic ars1. The signal intensities of the pEN53 and genomic ars1 bands were
Figure 4. Large fragments of human DNA can be propagated as SPARCs. Undigested high molecular weight DNA was prepared from 10 human genomic DNA \( \text{NotI} \) SPARC ura\(^+\) leu\(^+\) transformants, separated by PFGE and transferred by Southern blotting to GeneScreen and hybridized with \([\text{32P}]\text{CTP}\)-labelled total genomic human DNA. The resulting phosphorimage clearly shows that all of the transformants shown contain extrachromosomal linear SPARC ranging in size from \( \sim 100 \) to 500 kb. Several bands are visible in some transformants; it is likely that these result from rearrangements of the founding SPARC molecule.

The transformation efficiency of \( S.\text{pombe} \) protoplasts with small plasmids in the presence of Lipofectin was routinely \( 0.2-1 \times 10^6 \) transformants/\( \mu \)g input plasmid (19,20). It is difficult to estimate accurately the transformation efficiency with large heterogeneously sized molecules excised from the region of limiting mobility after ligation. Accurate transformation efficiencies can be derived by retransformation of \( S.\text{pombe} \) protoplasts with individual purified human SPARCs. SPARCs 2 (300 kb) and 8 (220 kb) were separated from endogenous chromosomes on low melting point gels and aliquots of the molten agarose slice containing only the SPARC were used to retransform FY562 protoplasts. The concentration of DNA in aliquots of the SPARC gel slices was estimated by comparison with a known amount of a single copy minichromosome Ch16-23R (550 kb, prepared from a known number of cells) (35) after separation by PFGE, blotting and hybridization with a labelled \( LEU2 \) probe (data not shown). Transformation of FY562 with 30 ng of SPARC 2 and 10 ng of SPARC 8 gave rise to 2035 and 1021 leu\(^+\) ura\(^+\) colonies, respectively. Thus the transformation efficiencies with these individual SPARCs were \( 1 \times 10^5 \) and \( 7 \times 10^4 \) transformants/\( \mu \)g input DNA.

Ten of the resulting SPARC 2 and 8 retransformants were analysed by PFGE to determine the state of the SPARC after this process (Fig. 7). Clearly three of the SPARC 2 and two of the SPARC 8 retransformants carry bands which are smaller than the original SPARCs, indicating that 20–30\% of such retransformants undergo rearrangements. It is likely that these rearrangements are induced by the transformation procedure itself.

DISCUSSION

The above data show that large fragments of human DNA can be cloned and propagated in fission yeast cells by using the SPARC vectors. Six different cosmids containing human DNA from chromosome 11p13 were replicated in fission yeast as multicopy acentric linear episomes without any rearrangement of the human DNA insert in any of the several independent transformants examined. In addition, large fragments from a total \( \text{NotI} \) digest of human genomic DNA were cloned directly into the SPARC system and propagated as multicopy large linear episomes of up to 450 kb.

The stability of the 10 SPARCs described has been monitored after propagation through two or three sequential rounds of propagation and recloning (not shown). In general, SPARCs were found to remain intact after 30, 60 and 90 divisions under selective conditions. However, occasionally smaller bands could be seen indicating that deletion events do occur during outgrowth. Such recloning experiments with SPARC 14 allowed the separation of the three bands detected in the original transformant (Fig. 4) into isolates containing single bands of 260, 425 and 470 kb. Analysis of 10 independent clones of SPARC 21 after \( \sim 60 \) divisions
Several of the SPARCS examined clearly contained FISH analyses produced consistent signals on chromosomes 18 chromosomes. SPARC 21 might contain a chimeric insert since found to map to a single location on metaphase spreads of human chimerism was observed with four of these since the insert was DNA–liposome aggregates by a single protoplast. It is possible that Lipofectin may isolates might result from co-tranformation events with independent frequency. Alternatively, the minor bands in primary SPARC rearrangements are not continually occurring at a very high propagation (up to 90 divisions) experiments, indicating that main and minor bands can be separated in recloning and an unstable founding molecule, the rearrangement of which leads derivatives of the main band or alternatively they might represent transformants is unknown. It is possible that these are rearranged retained the 90 kb band (not shown).

The origin of the minor bands in many of the SPARC transformants is unknown. It is possible that these are rearranged derivatives of the main band or alternatively they might represent an unstable founding molecule, the rearrangement of which leads to the formation of a more stable main band. In either case the main and minor bands can be separated in recloning and propagation (up to 90 divisions) experiments, indicating that rearrangements are not continually occurring at a very high frequency. Alternatively, the minor bands in primary SPARC isolates might result from co-tranformation events with independent recombinant SPARC molecules. It is possible that Lipofectin may act to promote the uptake of several SPARC molecules in recombinant SPARC molecules. It has many advantages when trying to detect particular recombinant DNA–liposome aggregates by a single protoplast.

Five SPARCs were examined by FISH and no evidence for chimerism was observed with four of these since the insert was found to map to a single location on metaphase spreads of human chromosomes. SPARC 21 might contain a chimeric insert since FISH analyses produced consistent signals on chromosomes 18 and 19. Several of the SPARCS examined clearly contained multiple bands (Fig. 4). Further examination of the SPARC 2 (300 kb) and SPARC 8 (220 kb) were utilized in gel purification/retransformation experiments to assess the transformation efficiency of S. pombe protoplasts with SPARC DNA (text). To determine if rearrangements are induced during the transformation procedure high molecular weight DNA was prepared from 10 (A) SPARC 2 (1–10) and (B) SPARC 8 (1–10) retransformants and separated by PFGE. For comparison DNA from the host strain FY562 and the primary SPARC 2 and SPARC 8 strains are included. Lanes M contain S. cerevisiae YPH724 as a marker with chromosomal sizes indicated. DNA in gels was visualized by staining with ethidium bromide. Note how clearly visible the SPARC DNAs are and that they migrate well away from all endogenous chromosomes (intense signal at limiting mobility region). Three SPARC 2 and two SPARC 8 retransformants contain minichromosomes that are significantly smaller than the original SPARC 2 and 8 molecules.

Figure 6. Estimate of the average SPARC copy number per cell. Restreaking and repicking single colony isolates allowed strains to be isolated containing only one predominant SPARC band. DNA was prepared from several strains containing SPARCs of the indicated size. SPARC 14-1 carries the 470 kb band while SPARCs 10-1 and 10-2 carry the 210 and 90 kb bands, respectively, detected by PFGE in Figure 4. Total genomic DNA from strains containing the SPARCs indicated was digested with CiaI, separated by conventional agarose gel electrophoresis and transferred by Southern blotting to GeneScreen and hybridized with \(^{32}P\)CTP-labelled 1.1 kb EcoRI ars1 gel-purified DNA fragment. CiaI releases the pEN53–LEU2–ars1–vector–telomere SPARC arm on a 5.3 kb fragment. The endogenous genomic ars1 locus is carried on a CiaI fragment of ∼18 kb. Comparison of the intensity of the SPARC pEN53-derived ars1 signal with that of the genomic ars1 band signal provides a convenient measure of the relative average copy number of the SPARC per cell. Most SPARCs examined were estimated to be at between 5 and 8 copies/cell. Control lanes show CiaI-digested DNA from the host strain FY562 and CiaI-digested pEN53-derived SPARC arm DNA. Signal intensities were measured using the ImageQuant software provided with the Molecular Dynamics PhosphorImager.
defined, these truncated centromeres are still ~15 kb, making any resulting vector arm cumbersome to manipulate (25–27). This is made even more difficult by the fact that these centromeric regions are difficult to clone and amplify in E.coli (25,26). Although SPARCs lack a centromere, they can be transmitted through meiosis in crosses with strains of the opposite mating type so that between 1 and 5% of progeny spores retain the intact SPARC (not shown). Presently, only the NotI site in the pEN51 and pEN53 SPARC arms can be utilized for cloning, but this can be easily modified by insertion of an appropriate adapter.

The method of transformation of *S. pombe* with large DNA requires the generation of protoplasts. The formation of *S. pombe* protoplasts is relatively easy to visualize since undigested cells are rod shaped with a length of 7–14 and a width of 4–5 μm whereas protoplasts are spherical. After transformation fission yeast protoplasts are plated by spreading on the surface of selective plates containing sorbitol to stabilize the osmotically sensitive protoplasts. The cell wall is regenerated efficiently under these conditions and does not require embedding in soft agar or other matrices, offering an advantage over most YAC protocols (2–6). The regeneration and growth of protoplast transformants on the surface of plates makes the colonies more manipulable. Replica plates of recombinant SPARCs can be easily generated by conventional means and colonies can be lifted directly onto filters, allowing immediate hybridization.

The relative high average copy number of SPARCs should facilitate the isolation of pure SPARC DNA in reasonable quantities for further experimentation. Since we have previously demonstrated that in fission yeast minichromosomes can be broken by telomere-mediated chromosome fragmentation (24), it is likely that SPARCs in *S. pombe* will be as manipulable as YACs in *S.cerevisiae*. Many selectable markers (e.g. *LEU2*, *ura4*, *ade6*+, *his3*+, *arg3*+, *lys1*+ and G418) are available for use in fission yeast strains auxotrophic for several supplements (30,36–39). The *ura4*+ gene is particularly useful since, like the *S.cerevisiae* URA3 gene, it can be counter selected in the presence of 5-fluoroorotic acid (24). This range of markers should allow the development of SPARC arms with different selectable markers and the same types of deletion by fragmentation or ‘retrofitting’ procedures to be performed on SPARCs that are currently applied to YACs (7,8,40,41).

Saccharomyces cerevisiae rad51, rad52 and rad54 mutant strains are defective in the process of homologous recombination and are utilized as hosts for YACs to minimize recombination, deletion and other rearrangements within or between YAC inserts (9,42–47). Analogous mutations in the *S.pombe* *rhp51*+, *rad22*+ or *rhp54*+ genes should have the same benefits for the SPARC system, although deletion of *rad22* (the RAD52 ortholog) only displays a 2-fold decrease in the frequency of homologous recombination compared with a 15-fold drop in strains with *rhp51* or *rhp54* gene deletions (48,49). Such strains might also be expected to reduce the frequency of chimeric SPARC clones (43).

We have previously demonstrated that chromosomes from *S. pombe* protoplasts can be transferred by PEG-mediated fusion into the nuclei of mouse tissue culture cells (50). This same type of procedure could be used to transfer SPARCs, modified to carry a suitable selectable marker, into tissue culture cells or even ES cells for the construction of transgenic mice. The use of YACs as a vehicle for transferring chromosomal regions of interest into somatic tissue culture cells, ES cells or mouse oocytes for the creation of transgenic animals is now well documented (51–60).

The large size of *S. pombe* endogenous chromosomes means that SPARCs should be more easy to gel purify away from host genomes in reasonable amounts for such purposes.

The SPARC cloning system described offers an alternative to the current large DNA cloning systems available. However, like all new technologies its true worth can really only be assessed with use and experience of others. We do not suggest that it will be a panacea for problems encountered by users of the *S.cerevisiae* YAC system, rather, we suspect that the SPARC system will be complementary, allowing some regions of target genomes to be propagated in fission yeast which are unclonable or prone to rearrangement in other systems. A total genomic library has not been constructed in SPARCs but this should involve no more effort than that required to construct YAC libraries.

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