MOLECULAR AND CELLULAR BIOLOGY, Sept. 1995, p. 5165–5172 Vol. 15, No. 9
0270-7306/95/$04.00+0

Replication of Centromere II of *Schizosaccharomyces pombe*

JESSICA G. SMITH,[1] MARK S. CADDLE,[1]† GABRIELLA H. BULBOACA,[1] JAY G. WOHLGEMUTH,[1] MARY BAUM,[2] LOUISE CLARKE,[2] AND MICHELE P. CALOS[1]*

Department of Genetics, Stanford University School of Medicine, Stanford, California 94305,[1] and Department of Biological Sciences, University of California, Santa Barbara, California 93106[2]

Received 13 December 1994/Returned for modification 14 February 1995/Accepted 26 May 1995

The centromeric DNAs of *Schizosaccharomyces pombe* chromosomes resemble those of higher eukaryotes in being large and composed predominantly of repeated sequences. To begin a detailed analysis of the mode of replication of a complex centromere, we examined whether any sequences within *S. pombe* centromere II (*cen2*) have the ability to mediate autonomous replication. We found a high density of segments with such activity, including at least eight different regions comprising most of the repeated and unique centromeric DNA elements. A physical mapping analysis using two-dimensional gels showed that autonomous replication initiated within the *S. pombe* sequences in each plasmid. A two-dimensional gel analysis of replication on the chromosomes revealed that the K and L repeat elements, which occur in multiple copies at all three centromeres and comprise approximately 70% of total centromeric DNA mass in *S. pombe*, are both sites of replication initiation. In contrast, the unique *cen2* central core, which contains multiple segments that can support autonomous replication, appears to be repressed for initiation on the chromosome. We discuss the implications of these findings for our understanding of DNA replication and centromere function.

The centromere is a specialized region of the chromosome that is involved in faithful chromosomal segregation during mitosis and meiosis. Specific proteins in conjugation with centromeric DNA make up the kinetochore or spindle attachment site, which, interacting with motor proteins and microtubules, orchestrates division of sister chromatids and directs individual chromosomes to the proper spindle pole. Centromeres of the budding yeast *Saccharomyces cerevisiae* have been well characterized (12) and consist of approximately 125 bp of unique DNA (13). In contrast, human centromeres are much larger, ranging in size from 300 to 5,000 kb (47, 48). Unlike *S. cerevisiae* centromeres, human centromeric DNA is highly repetitive. A major portion of human centromeric DNA consists of 171-bp repeats arranged in small and higher-order arrays of tandem repeats termed α-satellite DNA (48). This highly repetitive DNA is not transcribed and behaves as heterochromatin (reviewed in reference 48).

The centromeres of the fission yeast *Schizosaccharomyces pombe* more closely resemble human centromeres than do *S. cerevisiae* centromeres. *S. pombe*, whose symmetrical mode of cell division is similar to that of human cells, has a genome the same size as that of *S. cerevisiae* but only 3 chromosomes, as opposed to 16 for *S. cerevisiae*. *S. pombe* centromeres range in size from 40 to 100 kb and contain a 4- to 7-kb portion of unique or low-copy-number DNA designated the central core. The rest of the centromeric DNA consists of repeating units partially or entirely organized around the central core into a large inverted repeat (9, 11, 18, 20, 32, 33, 41). The centromere-specific repeats, which are probably not transcribed (18, 37), are organized into several different arrays, somewhat like the α-satellite repeats of human centromeres. Although a particular repeat unit may be found on more than one chromosome, the organization of repeat arrays is chromosome specific (11, 20, 32, 41) and varies among *S. pombe* strains (41). The core-associated repeats (CAR) that flank the central core at each centromere are chromosome specific (12, 20, 38). It has been shown recently that the central core region and a portion of the K repeat are necessary and sufficient to establish a functional centromere on a minichromosome in *S. pombe* (1).

We are interested in how *S. pombe* centromeres are replicated. Are they replicated from flanking sequences or from internal origins of replication? If internal origins exist, are they located within the portion of unique sequence or in one or more of the centromere-specific repeats? We have chosen to study *S. pombe* centromere II (*cen2*) because its central core region (cc2) consists entirely of unique DNA, facilitating analysis (11, 44).

Origins of DNA replication in *S. pombe* are not as well defined as they are in *S. cerevisiae*. In *S. cerevisiae*, replication origins were first identified by detection of their autonomous replication activity (22, 42, 43). *S. cerevisiae* autonomously replicating sequences (ARS) include an 11-bp highly conserved consensus sequence (7, 8, 49). Mutation at any one of the 11 bp can reduce replication efficiency (15, 46). ARS elements also have flanking regions that are required for full replication activity (8, 36). Two-dimensional (2-D) gel analysis (2, 23) can be used to physically map sites of initiation of replication. When the 2-D gel technique was applied to plasmids carrying ARS elements in *S. cerevisiae*, specific initiation was detected in the ARS region (2, 23). Most, but not all, *S. cerevisiae* ARS elements also mediate initiation in the chromosomes (14–16, 19), and these initiation sites are highly localized (14, 35, 36). In contrast to *S. cerevisiae*, autonomous replication in mammalian and *Drosophila* cells can be mediated by a large number of fragments (21, 25, 26, 39), and physical mapping techniques show that initiation is not highly localized (5, 24, 26). These observations suggest a lower level of sequence specificity for initiation of autonomous replication in higher eukaryotes.

To isolate origins of replication in *S. pombe*, a transformation efficiency assay has been used to identify fragments that can mediate autonomous replication (29). Among such fragments, a consensus sequence was found, but deletion of this sequence had no apparent effect on transformation efficiency (29). 2-D gel analysis was used to study a region near the *S.*

* Corresponding author. Phone: (415) 723-5558. Fax: (415) 725-1534. Electronic mail address: calos@leland.stanford.edu.

† Present address: Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA 02141.

pombe ura4 gene (17, 52). A weak initiation signal was detected over a region of approximately 4 kb and was found to correspond to several closely spaced, discrete origins. We have studied replication in *S. pombe* by using a transformation efficiency assay and 2-D gel analysis (6, 50). Our results show that initiation of autonomous replication is highly localized and apparently sequence specific, as is the case in *S. cerevisiae*. However, initiation at chromosomal origins appears to be less efficient and/or more dispersed in the *S. pombe* genome than in the *S. cerevisiae* genome.

Because of the availability of complete physical maps of *S. pombe* centromeric DNAs (11, 20, 41), combined with the ability to conduct physical mapping studies of replication in yeast cells, we have chosen to analyze replication in *S. pombe* centromeric DNA. Our study examines both the repetitive DNA and the unique cc2 region. We have determined where potential replication origins are located by using an autonomous replication assay and 2-D gel analysis. We also have determined which of the centromeric sequences containing potential origins are actual sites for replication initiation in the genome. The conclusions of this study provide insights into centromere structure and function and chromosomal DNA replication.

MATERIALS AND METHODS

Strains and plasmids. DNA for genomic 2-D gel electrophoresis was derived from strain 972h$^-$. The *ura4*$^-$ strain sp806 was used as the host for all yeast transformations. 972h$^-$ was grown in YEA medium, and sp806 was grown in Edinburgh minimal medium lacking uracil (31).

All plasmids are based on the YIp5 vector (43). Plasmid pY5-BJ was constructed by isolating a 6.1-kb *Nhe*I-*Kpn*I BJ insert from pSp1-N-b (11). *Hin*dIII linkers were added to the BJ fragment, and BJ was ligated to *Hin*dIII-digested YIp5. The L insert of pY5-L was isolated from pSp1-N-b by digestion with *Nhe*I and *Bam*HI. This 4.1-kb L fragment was ligated directly to *Nhe*I-*Bam*HI-digested YIp5, creating pY5-L. Two plasmids contain part of the K repeat; a 3.0-kb *Hin*dIII fragment isolated originally from tandem *cen3* K repeats (10) was ligated to *Hin*dIII-digested YIp5, creating pY5-3.0K, and a larger (6.4-kb) *Cla*I fragment, isolated from the same *cen3* K repeats, was ligated to *Cla*I-digested YIp5, creating pY5-6.4K. The BCAR insert is from a 2.4-kb *Bam*HI-*Hin*dIII digest of pSp3-SNc+K′ (1). *Hin*dIII linkers were added to BCAR, and it was ligated to *Hin*dIII-digested YIp5, creating pY5-BCAR. The 8.6 central core insert is an 8.6-kb *Nco*I-*Nco*I genomic fragment isolated from a *Bam*HI-*Sal*I digest of pSp-cc2 (1). The 8.6 insert was ligated to *Bam*HI-*Sal*I-digested YIp5, creating pY5-8.6. The 2.8 insert was isolated from a 2.8-kb *Spe*I-*Sph*I fragment of pY5-8.6. *Hin*dIII linkers were added to the 2.8 insert, and it was ligated to *Hin*dIII-digested YIp5, creating pY5-2.8. The 2.7 insert is a 2.7-kb genomic *Nco*I-*Eco*RI fragment isolated from a *Bam*HI-*Eco*RI digest of pSp-cc2 (1). The 2.7 insert was ligated to *Bam*HI-*Eco*RI-digested YIp5, creating pY5-2.7. The 1.3 insert is a 1.3-kb *Cla*I-*Sph*I genomic cc2 fragment that was cloned into another vector and then digested with *Bam*HI and *Sph*I. This *Bam*HI-*Sph*I fragment was ligated to *Bam*HI-*Sph*I-digested YIp5, creating pY5-1.3. For pY5-1.5, the 1.5 insert is a 1.5-kb *Sna*BI-*Nco*I genomic fragment. *Hin*dIII linkers were added to the 1.5 fragment, and it was ligated to *Hin*dIII-digested YIp5. The 2.7, 1.3, 2.8, and 1.5 inserts are all subclones of the larger 8.6 insert.

Yeast transformations were performed by an electroporation protocol (38). One microgram of each plasmid was transformed.

Yeast DNA isolation, 2-D gel electrophoresis, and hybridization. All procedures were carried out as previously described (6).

Computer analyses. Quest 5.4 (IntelliGenetics) was used to search for the 11-bp consensus sequence [(A/T)(A/G)TTTATTTA(A/T)] found by Maundrell et al. (29). Three genomic *Hin*dIII fragments were tested. The accession numbers for the original *Hin*dIII genomic clones, named pYC116, pYC111, and pSS113, are X66741, X66739, and X13764, respectively (9, 44). Two of these *Hin*dIII genomic fragments are internal to the cc2 and CAR regions, while the third is identical to the 3.0K fragment. The fragments included in the Quest search include the 3.0K, 2.7, 1.3, 1.5 fragments and most of the 2.8 and 8.6 fragments.

RESULTS

Transformation with 10 cloned DNA segments from the *cen2* region into *S. pombe* yielded 9 that confer a high transformation efficiency. We created a series of 10 plasmids containing genomic fragments from the *cen2* region of *S. pombe* cloned into the vector YIp5 (43). None of these segments alone is sufficient to establish a functional centromere in *S. pombe* (1). YIp5 carries the *URA3* gene from *S. cerevisiae*, which serves as a selectable marker in *S. pombe* by complementing a mutation in the *S. pombe ura4* gene. Cloning details are discussed in Materials and Methods. Figure 1 depicts *cen2* and the fragments that were cloned. These 10 plasmids, along with the vector control, YIp5, were transformed into the *S. pombe ura4*$^-$ strain sp806. The resulting transformation efficiencies are listed in Table 1. Nine of the transformation efficiencies were high and similar to those that corresponded in previous studies to the presence of strong autonomous origins of replication (6, 50). In addition to reporting transformation efficiencies in Table 1, we also note the sizes of individual transformant colonies. Only one plasmid from the *cen2* series, pY5-6.4K, had a low efficiency of transformation, producing only a few very small colonies. It is interesting that a plasmid carrying a subclone of this region, pY5-3.0K, showed a high transfor-

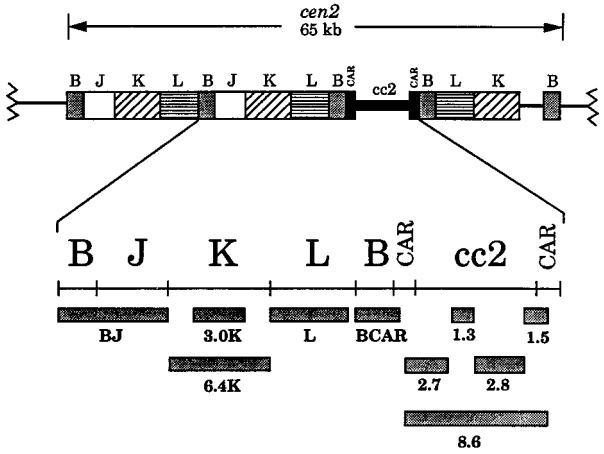

FIG. 1. Chromosomal map of *cen2* of *S. pombe* (strain 972h$^-$) and locations of the cloned *cen2* segments used in this study. *cen2*, which has been previously described in detail (1, 9, 11, 18, 41, 44), consists of the unique cc2 region with a chromosome-specific CAR on each side, flanked by several centromere-specific repeating sequences termed B, J, K, and L. The repeats are organized into a large inverted repeat around cc2. The 10 *cen2* genomic fragments used in this study are indicated below the map (gray boxes). All genomic inserts were cloned into the vector YIp5 (see Materials and Methods for details). Plasmids named with letters correspond to the repeat sequences that they contain. Plasmids named with numbers correspond to the sizes of the inserts.

TABLE 1. Transformations with 10 cloned segments from *cen2* into *S. pombe*

Plasmid	Transformation efficiency[a]	Colony size[b]
YIp5	0	
pY5-BJ	1,764	Large and small
pY5-3.0K	2,968	Large
pY5-L	1,988	Large and small
pY5-BCAR	2,170	Large
pY5-8.6	1,316	Large
pY5-2.7	2,086	Large
pY5-1.3	1,820	Large and small
pY5-2.8	1,694	Large
pY5-1.5	2,408	Very small
pY5-6.4K	228	Very small

[a] Expressed as the approximate number of colonies produced per microgram of plasmid DNA.
[b] Determined after 5 days of growth at 30°C.

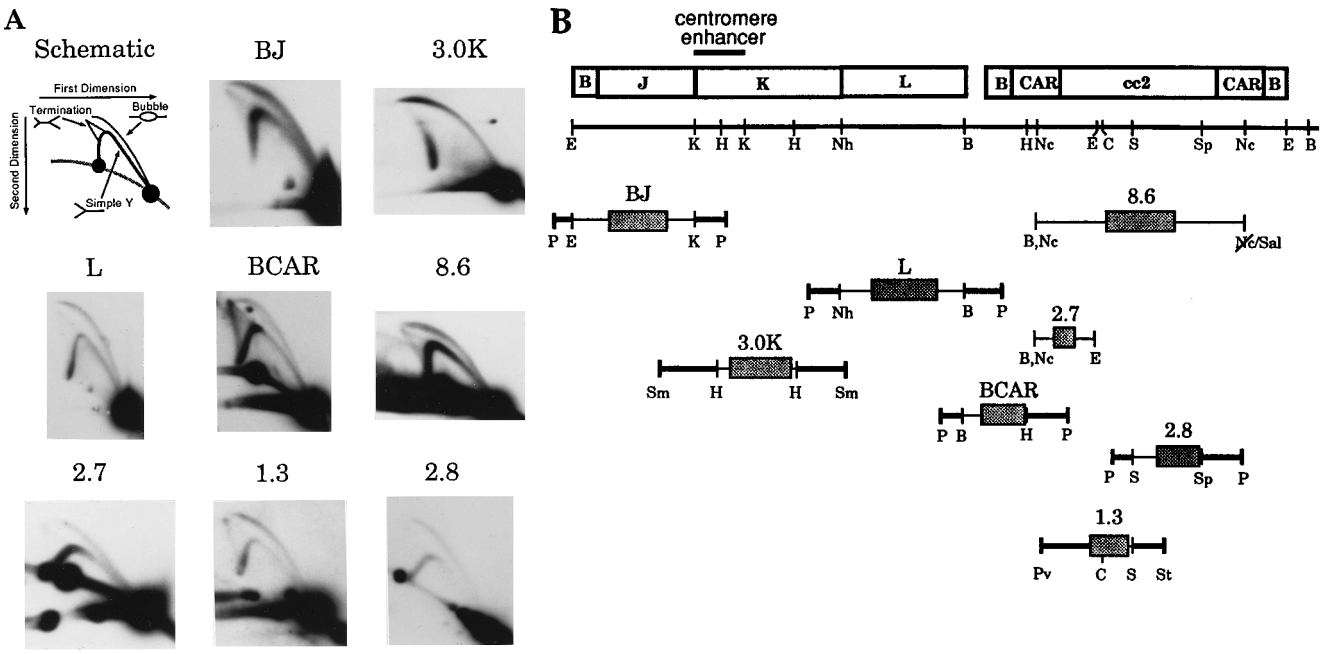

FIG. 2. 2-D gel analysis of autonomously replicating plasmids in *S. pombe*. Total DNA samples from eight actively growing yeast transformants, each containing one of the plasmids which had a high transformation efficiency and large colony size in the transformation assay, were analyzed by 2-D gel electrophoresis. (A) The first panel depicts a schematic diagram of 2-D gel patterns. This diagram is a guide to interpreting the results from the 2-D gels run in this experiment. The other panels depict the autoradiographic results from 2-D gel analysis. (B) The linear map locates where the inserts that were cloned into YIp5 exist in *cen2*. In general, the outer restriction sites of each fragment indicate which enzymes were used for 2-D gel analysis. The inner sites locate the genomic insert that was cloned into YIp5. Thick black lines represent vector sequences used as a probe, and thin black lines represent genomic inserts. The gray boxes represent the bubble detection zones, which are the middle one-third of each fragment used for 2-D gel analysis. In the cases of the 8.6 and 2.7 fragments, there are no vector sequences shown because the insert was used as a probe. BJ, *Pst*I-digested DNA probed with a 2.4-kb *Pst*I-digested YIp5 fragment; 3.0K, *Sma*I-digested DNA to linearize YIp5-3.0K, probed with full-length YIp5 vector; BCAR, *Pst*I-digested DNA probed with a 2.4-kb *Pst*I-digested YIp5 fragment; 8.6, *Bam*HI- and *Sal*I-digested DNA to isolate the *Nco*I-*Nco*I 8.6 fragment and probed with full-length 8.6-kb insert; 2.7, *Bam*HI- and *Eco*RI-digested DNA to isolate the *Nco*I and *Eco*RI 2.7 fragment and probed with full-length 2.7-kb insert; 1.3, *Pvu*II- and *Stu*I-digested DNA probed with a 4.2-kb *Pvu*II- and *Stu*I-digested YIp5 fragment; L, *Pst*I-digested DNA probed with a 2.4-kb *Pst*I YIp5 fragment; 2.8, *Pst*I-digested DNA probed with a 2.4-kb *Pst*I YIp5 fragment. Exposure times varied from less than 1 day to 9 days, except for pY5-1.3, which required a 3-week exposure. The centromere enhancer is a 2.1-kb *Kpn*I fragment located within the K repeat element. E, *Eco*RI; K, *Kpn*I; H, *Hin*dIII; Nh, *Nhe*I; B, *Bam*HI; Nc, *Nco*I; C, *Cla*I; S, *Sph*I; Sp, *Spe*I; Pv, *Pvu*II; St, *Stu*I; Sm, *Sma*I; P, *Pst*I; Sal, *Sal*I.

mation efficiency; sequences inhibitory to replication are apparently present in the larger fragment. pY5-1.5, although positive with respect to transformation efficiency, yielded very small colonies. Cells from these small colonies grew slowly in liquid culture and never reached the optimal density for 2-D gel analysis (see below).

Transformants derived from plasmids giving a high transformation efficiency were analyzed further. Whether the plasmids were autonomous or integrated was determined by digesting DNA extracted from each transformant with *Not*I, which does not have a recognition site in any of the plasmids. An autonomous plasmid is expected to run as a supercoiled band below the *Not*I-digested chromosomal band. *Not*I digests and Southern analysis confirmed that all of the plasmids existed as autonomous replicons and were not integrated into the genome. Although the DNA from most replicons consisted of monomeric supercoiled, nicked, and some linear DNA, there were also detectable signals from dimers and higher-multimer plasmids from most transformants. The presence of multimeric forms has been frequently observed in *S. pombe* (6, 30, 44, 50, 51) and may result from homologous recombination. pY5-BCAR, pY5-BJ, and pY5-L transformants contained a significant amount of plasmid dimers and multimers. Plasmid pY5-1.5 was detectable only in a dimeric form (see below). In summary, we determined that most fragments derived from *cen2*, when cloned into YIp5, were able to produce high transformation efficiencies consistent with autonomous replication. We further ascertained that these *cen2* plasmids exist as autonomous, unintegrated elements, which is also consistent with the interpretation that most of the *cen2* fragments tested can mediate autonomous replication.

2-D gel analysis of autonomously replicating plasmids. We used 2-D gel analysis to verify that the plasmids were replicating autonomously and to determine whether initiation of autonomous replication was originating from the *cen2* inserts. Total DNA was extracted from actively growing yeast strains, each carrying a plasmid that yielded a high transformation efficiency. The DNA from each transformant was first digested with restriction enzymes that released the *cen2* insert and, in some cases, parts of YIp5. We then subjected the digested DNA to 2-D gel analysis (2), blotted the resulting gels, and probed the blots with specific ^{32}P-labeled DNA fragments. Probes that eliminated or significantly minimized the amount of hybridization from the genomic copies of the repeats were selected. Figure 2A shows a schematic diagram of 2-D gel patterns and the resulting autoradiographs from 2-D gel analysis for eight of the nine high-transformation-efficiency clones. Figure 2B locates the *cen2* inserts in the genome and shows the digests and probes used for 2-D gel analysis and the resulting bubble detection zone, representing the middle one-third of the fragment probed.

Three basic types of replication intermediates that can exist in a restriction fragment are a simple Y, a bubble, and a double Y or termination signal (Fig. 2A, schematic). A simple Y is

formed by the presence of a single replication fork passing through the fragment. A bubble occurs when two replication forks are present in the fragment and are moving away from each other. The presence of a bubble indicates that replication initiated within the given restriction fragment. A termination signal, or double Y, is also formed by two replication forks in the fragment, but in this case the forks are moving towards each other until they ultimately meet at a replication terminus.

Transformant DNA containing pY5-BJ was digested with *Pst*I to release the BJ insert plus 2.1 kb of YIp5 DNA distributed on both sides of the insert (Fig. 2B, BJ fragment). After 2-D gel analysis, the blot was probed with the ^{32}P-labeled 2.4-kb *Pst*I fragment of YIp5 DNA, so that the probe would hybridize only to plasmid and not chromosomal DNA (Fig. 2A, BJ). A strong bubble signal was clearly visible, indicating that a major initiation site is contained within the BJ insert. We also detected a small amount of complete simple Y signal that was not attributable to an asymmetric bubble pattern. A faint termination signal was also present. In the case of BJ and several of the other transformants, we attributed the presence of complete simple Y and weak termination signals to the presence of plasmid dimers and higher multimers in which only one origin fired, leading to simple Y or termination signals over the unused origins that would also be detected by the probe. It is known that initiation on *S. cerevisiae* plasmids containing more than one origin is usually confined to one origin on a given molecule (3). We have made similar observations in *S. pombe* (50). The simple Y and termination signals could also be due to the presence of more than one origin within a *cen2* fragment. This situation would give rise to a bubble signal when the origin closest to the center of the fragment fired and a simple Y signal when the other origin(s) fired. A termination signal would be present in the rare case in which both origins initiated on the same plasmid (3).

The extracted DNA containing pY5-3.0K was cut with *Sma*I. The *Sma*I site in YIp5 linearizes the pY5-3.0K plasmid and places the 3.0K insert approximately in the middle third of the plasmid (Fig. 2B, 3.0K fragment). The blot resulting from 2-D gel analysis was probed with ^{32}P-labeled YIp5, and the autoradiograph shows a strong bubble signal (Fig. 2A, 3.0K). The presence of this signal indicates that replication is initiating only at a site near the center of the 3.0K fragment.

DNA from cells containing pY5-BCAR was digested with *Pst*I (Fig. 2B, BCAR fragment), and the blot from the 2-D gel was probed with the same ^{32}P-labeled *Pst*I 2.4-kb YIp5 fragment as was used for the BJ panel. The resulting autoradiograph shows a bubble signal, indicating that replication initiated within the BCAR fragment (Fig. 2A, BCAR). The faint Y and the termination signals can be attributed to the presence of dimers and other multimers in the pY5-BCAR transformant. It is also possible that other origins are located within this 2.4-kb fragment. DNA from cells containing pY5-L was digested with *Pst*I (Fig. 2B, L fragment), and the resulting 2-D blot was probed with the ^{32}P-labeled 2.4-kb fragment of YIp5 DNA. The result showed a strong bubble signal indicating initiation from within fragment L (Fig. 2A, L). The accompanying Y signal could be due to the presence of plasmid multimers or to additional origins within L.

Extract from cells containing pY5-8.6 DNA was digested with *Bam*HI and *Sal*I to release the 8.6-kb insert from the polylinker of the vector (Fig. 2B, 8.6 fragment). The full 8.6-kb insert was ^{32}P labeled and used as a probe. Because the exposure time was only 20 h for this panel, we are confident that the signals detected are from the autonomous plasmid. A much longer exposure time is required to visualize replication signals from the *S. pombe* genome (see below). The presence of a strong bubble signal demonstrates that there is an initiation site located near the center of the 8.6-kb fragment. The simple Y signal and faint termination signal are expected from the presence of at least two other asymmetrically placed initiation sites within the 8.6-kb fragment (see below). Southern analysis detected only a very faint signal from plasmid multimers; thus, it is likely that the bubble and Y signals are attributable solely to these multiple origins.

Portions of the 8.6-kb *cen2* fragment were subcloned as the 2.7, 1.3, 2.8, and 1.5 fragments. DNA containing plasmid pY5-2.7 was digested with *Bam*HI and *Eco*RI, which excise the 2.7-kb insert from YIp5 (Fig. 2B, 2.7 fragment). The entire 2.7-kb fragment was used as a ^{32}P-labeled probe for the resulting blot. Again, the minimal exposure time indicated that the replication signals observed derived predominantly from plasmid DNA. The 2.7 panel in Fig. 2A clearly shows a bubble signal, indicating the presence of an initiation signal within the 2.7-kb fragment subcloned from the 8.6-kb fragment.

We also confirmed the presence of an initiation site within the 1.3-kb fragment (Fig. 2A, 1.3). This DNA was digested with *Pvu*II and *Stu*I, which are both unique sites within YIp5 (Fig. 2B, 1.3 fragment). This digest left 2.7 and 1.5 kb of vector DNA flanking the 1.3-kb insert. The ^{32}P-labeled fragment used as a probe was YIp5 DNA containing no insert and lacking the *Pvu*II-*Stu*I piece excised in the digest. For this insert, a long (21 days) autoradiographic exposure time was necessary, but because the probe consisted only of YIp5 DNA, we were assured of detecting only the autonomous plasmid DNA. DNA from cells containing pY5-2.8 was digested with *Pst*I, and the ^{32}P-labeled 2.4-kb *Pst*I YIp5 DNA probe was used (Fig. 2B, 2.8 fragment). A strong bubble signal was detected (Fig. 2A, 2.8), indicating a major initiation site within the 2.8-kb fragment. Therefore, we have detected three distinct, strong replication initiation sites (2.7, 1.3, and 2.8) located within the 8.6-kb fragment.

2-D analysis was also performed on the transformant containing pY5-1.5. This plasmid produced a high transformation efficiency, but the small colonies never grew in liquid culture to a density optimal for 2-D gel analysis. DNA from cells containing pY5-1.5 was digested with *Pst*I, and the resulting blot was probed with the ^{32}P-labeled *Pst*I 2.4-kb fragment of YIp5 DNA. After long exposure, the autoradiograph showed a barely detectable bubble signal, with the majority of the signal present as a simple Y pattern (data not shown). This Y signal cannot be attributable to chromosomal signal, because only vector DNA was used as a probe. From Southern analysis, we know that plasmid pY5-1.5 is detectable only as dimers and higher multimers, suggesting that a single copy of the 1.5 fragment was inadequate to mediate efficient replication. We concluded from these data that the majority of the time, the 1.5 fragment is not able to act as a replication origin, explaining the small size of the colonies and the weak bubble signal. The other eight centromeric DNA segments, however, clearly contain sequences that function as efficient replication origins when carried on plasmids on *S. pombe*. Three of the origin fragments are contained within the 8.6-kb fragment. Furthermore, as noted, we cannot exclude that the BJ, BCAR, and L fragments contain more than one origin. However, as noted above, the pY5-BCAR, pY5-BJ, and pY5-L transformants did contain multimeric plasmid forms, and thus the mixed bubble and Y 2-D gel patterns could be due to simply these forms rather than to additional origins in each fragment. Therefore, a minimum of seven different origins are contained in the *cen2* region of DNA.

2-D gel analysis of genomic *cen2* sequences. We have shown that most fragments from the *cen2* region of *S. pombe* can

mediate autonomous replication and that replication initiates from at least one site within the cloned *cen2* fragment in each case. It is known from results for *S. cerevisiae* that not all origins that function as ARS elements give rise to replication on the chromosomes (14–16, 19). Therefore, we were interested to know how many of the *cen2* sequences that can initiate autonomous replication on a plasmid can also initiate replication from this highly specialized chromosomal location. *S. pombe* genomic DNA from actively growing yeast cells lacking plasmid DNA was digested in seven separate reactions. The digested DNA was then subjected to 2-D gel analysis. Seven different probes were used to span most of the genomic sequences covered by the eight plasmids that showed strong initiation in Fig. 2.

The 2-D gel analyses of the K, L, cc2, and CAR genomic *cen2* sequences are shown in Fig. 3. To analyze replication from the K repeat, DNA was digested with *Hin*dIII, and fragment I was used as a probe for the resulting blot after 2-D gel analysis. Because of the multiplicity of K sequences from the centromeres of chromosome 1 (2 copies of K), chromosome 2 (3 copies), and chromosome 3 (approximately 12 copies), background signal was detected. However, a clear bubble pattern is visible (arrows in Fig. 3I). Panel B of Figure 3I is a darker exposure of panel A to illustrate the bubble pattern more clearly. It is highly unlikely that this bubble signal is actually a simple Y pattern from a partial *Hin*dIII digestion because the origin of the bubble signal can be extrapolated to the 1X spot where the Y signal originated and the bubble also has the proper shape for a bubble rather than a Y signal. In addition to the bubble pattern, a Y pattern is also detectable. We infer from Fig. 3I that the K repeats are able to initiate replication in the genome but that not every individual K repeat is initiating in every cell cycle. In addition, it is not yet known whether all copies of the resident K repeats are required for full function of each centromere in *S. pombe* (1). We surmise that most of the numerous K repeats are replicated passively from external origins, accounting for the strong simple Y signal. However, because this 2-D pattern is a mixture of Y and bubble signals, we cannot rule out the possibility that there are multiple origins within the K repeat. Multiple origins seem unlikely, however, because the plasmid 2-D gel analysis of K showed a typical asymmetric bubble pattern, arguing that there is only one origin in this region. However, it remains possible that initiation in a chromosomal context is more dispersed than initiation of autonomous replication. Alternatively, replication bubbles may be more susceptible to breakage at one fork in chromosomal DNA than in plasmids.

To analyze cc2, which is necessary but not sufficient for centromere function (1), fragment II was used as a probe for *Bam*HI-digested DNA subjected to 2-D gel analysis (Fig. 3II). The pattern observed was unique and did not correspond to that of the typical replication intermediates. We saw a similar pattern when *Nco*I-digested DNA subjected to 2-D gel analysis was probed with fragment III (Fig. 3III). For these larger fragments, we used gel running conditions that we previously adapted for large fragments (24). We hypothesized that the unique patterns observed were due to an interaction in vitro between the two CAR sequences present in these fragments that might distort mobility of the fragment, especially under the second-dimension gel running conditions.

To avoid this problem, we digested genomic DNA with *Nde*I and *Spe*I and probed the resulting 2-D blot with fragment IV (Fig. 3IV). Fragment IV represents unique cc2 DNA that contains no CAR sequences. The resulting autoradiograph showed a simple Y sequence with neither detectable bubble signal nor the unusual pattern seen with the fragments containing two CAR sequences. We concluded from Fig. 3IV that there is no detectable initiation from within the cc2 region in the genome, even though fragment IV contains the 1.3- and 2.8-kb segments that initiate efficient autonomous replication.

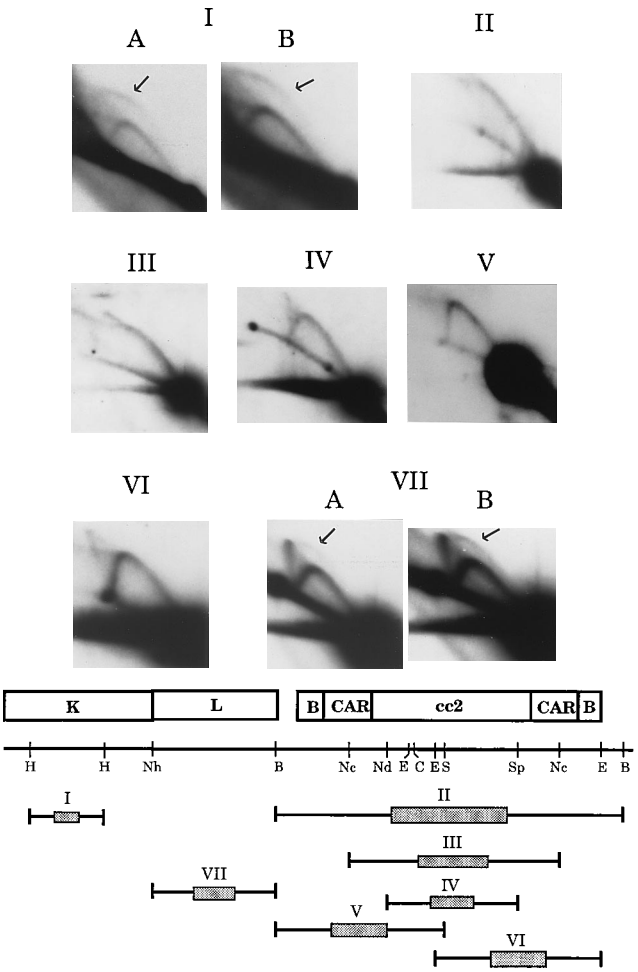

FIG. 3. 2-D gel analysis of genomic sequences of *cen2*. Total DNA was collected from the actively growing yeast strain 972h$^-$ and subjected to 2-D gel analysis. The top panels depict the results from the autoradiographs. The fragments below the chromosomal map of *cen2* indicate the genomic fragments probed. (I) *Hin*dIII-digested DNA probed with the 3.0-kb fragment I. Panel B is a longer exposure of panel A. (II) *Bam*HI-digested DNA probed with the 5.0-kb unique *Nde*I-*Spe*I DNA fragment internal to fragment II. (III) *Nco*I-digested DNA probed with fragment III. (IV) *Nde*I- and *Spe*I-digested DNA probed with fragment IV. (V) *Bam*HI- and *Sph*I-digested DNA probed with a unique 3.0-kb *Sna*BI-*Sph*I fragment internal to fragment V. (VI) *Eco*RI-digested DNA probed with a 1.1-kb *Sph*I-*Sna*BI fragment and a 1.9-kb *Sna*BI fragment. Both probes consist of unique sequences internal to fragment VI. (VII) *Nhe*I- and *Bam*HI-digested DNA probed with fragment VII. Panel B is a longer exposure of panel A. The arrows depicted in panels I and VII locate the bubble signals. In the linear map, the gray box in the center third of each fragment designates the bubble detection zone. H, *Hin*dIII; Nh, *Nhe*I; B, *Bam*HI; Nc, *Nco*I; Nd, *Nde*I; E, *Eco*RI; C, *Cla*I; S, *Sph*I; Sp, *Spe*I.

To examine replication patterns from a fragment containing only one CAR sequence, we digested genomic DNA with *Bam*HI and *Sph*I. The resulting 2-D blot was probed with fragment V, which contains only one CAR sequence. As shown in Fig. 3V, the result was a simple Y signal with no detectable bubble pattern. This result indicated that replication proceeds passively through this fragment, even though fragment V includes the 2.7-kb sequence that initiates efficient autonomous replication. We also noticed that the presence of only one

CAR sequence eliminated the unusual pattern seen with probes containing both CAR sequences.

We digested DNA with *Eco*RI and probed with fragment VI in order to examine the other half of cc2 (Fig. 3VI). Again, with a probe containing only one CAR sequence, we detected a simple Y pattern and no bubble signal, indicating that passive replication was also occurring through this fragment. Fragment VI includes the 2.8-kb region of unique cc2 sequence that gives efficient autonomous replication; the same fragment showed no initiation in the genome.

To analyze the L repeat unit, we digested DNA with *Nhe*I and *Bam*HI and probed the resulting 2-D blot with fragment VII (Fig. 3VII). As was the case with fragment I, panel B in Fig. 3VII is a longer exposure to demonstrate the bubble pattern more clearly. As with the K repeat, the repetitive nature of the L repeat caused a large background signal. We were able to detect a bubble signal, as well as termination and Y patterns. We concluded that replication initiates within the L repeat. It is highly unlikely that this bubble signal is actually a simple Y pattern resulting from partial digestion for the same reasons described above for the K repeat. As noted for the K repeat, apparently every L repeat does not initiate in each cell cycle. However, we similarly cannot rule out that replication occurs in a broad zone over the L repeat.

To analyze replication from the B and J repeat units, DNA was digested with *Cla*I and *Hin*dIII (11). This digest places all of the B repeat and one-third of the J repeat in the middle third of the fragment. The resulting blot from 2-D gel analysis was probed with the entire *Cla*I-*Hin*dIII genomic fragment. Because the *cen2*-specific B repeat is not always adjacent to the J repeat, the *cen2* genomic fragments that hybridize to the BJ probe are of varied sizes (1, 11, 41). The resulting 2-D analysis detected several weak simple Y signals (data not shown) presumably caused by hybridization to a mixture of *cen2* B repeat fragments. There was not enough signal present to determine whether a bubble pattern occurred within the B or J repeats.

Therefore, considering all of the panels in Fig. 3, we conclude that the K and L repeats are the only *cen2* regions tested from which replication initiation could be detected in the chromosome. The 8.6, 2.7, 1.3, 2.8, and CAR sequences did not contain detectable chromosomal origins. Because of the variability of B and J, we were unable to determine if there was chromosomal initiation from these regions.

Sequence analysis of regions of *cen2*. A transformation efficiency assay similar to the one described in this study was used by Maundrell et al. (29) to find sequences which could support autonomous replication in *S. pombe*. From among these sequences, an 11-bp consensus sequence was identified (29). However, the deletion of this 11-bp sequence seemed to have no effect on transformation efficiency, leaving open its significance. We used the Quest 5.4 program to search for the 11-bp consensus sequence within several *cen2* fragments (9, 44) that exhibited autonomous replication in this study. We examined the 3.0-kb portion of the K repeat and portions of the cc2 region, including the 2.7, 1.3, and 1.5 fragments and parts of the 2.8 fragment. We found one match to the consensus within the 3.0K fragment that corresponds to the consensus sequence reported previously for *cen1* (44) and two matches within the 2.8 fragment. However, we did not find a match to the consensus sequence in the 2.7, 1.3, or 1.5 fragment. Therefore, we did not find a direct correlation between autonomous replication and the consensus sequence defined by Maundrell et al. (29).

DISCUSSION

We have investigated the ability of centromeric DNA sequences from chromosome II of *S. pombe* to replicate autonomously and to initiate replication in the chromosome. To test autonomous replication, we created a series of plasmids containing genomic restriction fragments from the *cen2* region. In a transformation efficiency assay, we found that 9 of 10 tested plasmids had a high transformation efficiency. Eight of these nine plasmids also produced sizable transformed colonies and mediated efficient autonomous replication. To confirm that initiation was originating from these *cen2* DNA segments, we performed 2-D gel analysis on all of the efficiently autonomously replicating plasmids and confirmed that all of them had at least one strong initiation site located within the *cen2* insert.

Therefore, we have found eight efficient ARS elements from the *cen2* region corresponding to at least seven different plasmid-borne origins of replication. We cannot rule out the possibility that some of these *cen2* fragments contain multiple initiation sites, as is definitely the case for the 8.6-kb fragment. The existence of at least seven initiation sites in a 29-kb region indicates that *S. pombe* centromeric DNA contains a higher density of potential origins than has been demonstrated for random *S. pombe* genomic DNA, where ARS elements occur every 20 to 50 kb (6, 29, 50). Multiple potential origins within a small region also have been reported in *S. pombe cen1*, as determined in a similar transformation assay (44). In this case, overlapping genomic clones from *cen1* were tested, and 9 of 13 clones showed a high transformation efficiency. Considering that the clones were overlapping, this result reflected a minimum of four ARS elements within a 44-kb area. Other cases of closely spaced origins have been observed in *S. pombe* in noncentromeric DNA (50, 52), but no other large regions with such high origin densities have been characterized to date in nontandemly repeated regions.

We carried out genomic 2-D gel analysis on the *cen2* region to determine which of the sequences that can initiate replication on a plasmid are also able to do so in a chromosomal context. Our results showed an initiation signal occurring in the K and L repeats. Because we also saw a strong simple Y pattern in these genomic 2-D autoradiographs, it seems likely that not all K and L repeats initiate in a given cell cycle. We estimate from the autoradiographs of these repeats that approximately 5 to 10% of the total signal is due to initiation events. This situation is similar to that in the ribosomal repeats of *S. cerevisiae*, in which a mixed Y and bubble signal is seen over the ribosomal DNA ARS element (4, 27). In *S. cerevisiae*, this result has been interpreted to mean that only a minority (1 in 3 to 1 in 10) of the ribosomal DNA ARS elements are used to initiate replication (27). Interestingly, there is an ARS element (*ARS 308*) associated with *CEN3* of *S. cerevisiae* that functions as an inefficient chromosomal replication origin (20). *S. pombe* centromeric replication also bears some resemblance to the replication of human centromeres, in which it has been recently demonstrated that replication initiation appears to occur at a small minority of the alphoid sequences that comprise the bulk of the human centromere (45). In this regard, it is interesting that the predominant repeat elements in both *S. pombe* and human centromeres have the capacity to initiate replication.

The genomic data that we have presented cannot make an absolute distinction between localized initiation in a subset of K or L repeats versus dispersed initiation in each K or L repeat. We favor the former interpretation because initiation mediated by isolated K or L repeats, as well as other *S. pombe* origins, on plasmids is localized (Fig. 2) (6 and 50). To rule out

dispersed initiation on the chromosome, the 2-D gel patterns of the fragments adjacent to bubble-containing fragments should be examined. These experiments have not proven to be feasible in the centromeric region because of the repetitive nature of K and L and the variety of different fragments that adjoin them. Such studies should be pursued in single-copy origin sequences, for which a mixed bubble and Y pattern is also consistently observed (6, 17, 50, 52).

That the K repeat can initiate replication in the *S. pombe* genome is of interest, because the DNA requirements for a functional centromere in *S. pombe* comprise central core sequences and a section of the K repeat that overlaps the portion of K that we have tested here (1). Within the 2.1-kb segment of the K repeat that is essential for centromere function (1) is a novel *cis*-acting DNA element termed a centromere enhancer (Fig. 2B), which alters central core chromatin structure at a distance and in an orientation-independent and functionally relevant manner (28). We do not yet know whether the centromere enhancer and replication origin activities include the same DNA sequences, but clearly they are in close proximity. In any case, the sole function of the K repeat cannot be to serve as an origin of replication, because it has been demonstrated that the L repeat, a portion of which also functions as an origin in the chromosome, cannot substitute for K in establishing centromere function (1).

We concluded that the fragments comprising the cc2 and CAR regions of *cen2*, which acted as efficient ARS elements, apparently do not act as efficient chromosomal origins. It is perhaps surprising that so many potential origins were not active in a chromosomal context. Several studies using *S. cerevisiae* have reported sequences that function as ARS elements on plasmids but are unused or inefficiently used in their chromosomal contexts (14–16, 19). It has been postulated that these ARS elements are silent in the chromosome because these sequences, or the regions of DNA surrounding these sequences, are involved in other interactions or functions that do not allow these origins to initiate replication.

The central core region may fail to initiate replication efficiently in the chromosome because it has other centromere-related functions. The chromatin comprising the cc2 and CAR regions is nuclease resistant, implying an unusual packaging form for this region that is devoid of regularly spaced nucleosomes (37). This unusual chromatin conformation of the central core correlates with centromere function (28). In contrast, the centromere region containing the repeat units is nuclease sensitive and appears to be regularly packaged into nucleosomes like bulk *S. pombe* chromatin. A model has been suggested wherein the K repeat, containing the centromere enhancer, interacts with the central core region by DNA looping to effect a functional centromere (10, 28). It has also been hypothesized that this interaction would create a higher-order structure that might in some way be required for centromere function (10, 37, 40). If this model is correct, the DNA packaged in this higher-order structure might not be accessible to the replication proteins required to initiate DNA synthesis, either because of a steric hindrance or because of an alternative function, such as the recruitment of centromere-specific proteins required for centromere function.

Attempts have been made to define a consensus sequence for origins of replication in *S. pombe* (29, 44). The significance of the 11-bp consensus sequence defined by Maundrell et al. (29) is unclear. Another study looked for sequence homologies to this 11-bp sequence in *cen1* but found no direct relationship between the consensus sequence and a high transformation efficiency (44). We searched several of our fragments for homology to this consensus sequence. We found the sequence in two DNA fragments that could support autonomous replication, but not in three other DNA fragments that could also do so. We have found the consensus sequence in the K repeat, a region that we have determined can also initiate replication in the chromosome. However, we have also found that the 2.8-kb region of cc2 that contains two consensus sequences does not initiate replication in the chromosome. The consensus sequence contained within the K repeat unit is located outside the 2.1-kb K fragment that contains the centromere enhancer (28) and that Baum et al. (1) find, in conjunction with the central core region, to have centromere activity.

Replication origins in *S. pombe*, although initiating from a fixed site, may encompass a larger area than those of *S. cerevisiae*, which consist of the consensus sequence and approximately 100 to 200 bp of flanking DNA (34). Maundrell et al. (29) were unable to clone autonomously replicating fragments smaller than 800 bp. A gross deletion analysis of an *S. pombe* origin characterized by Caddle and Calos (6) revealed that approximately 1.0 kb of DNA was required for full replication efficiency. An *S. pombe* origin of replication may require signals other than a consensus sequence, and these signals may be more spread out than the required flanking regions of *S. cerevisiae*. The *S. pombe* consensus sequence might form part of a potential chromosomal origin, but this region might not initiate replication unless other DNA signals are also present.

The K and L repeats make up 70% of the total centromeric mass of DNA in *S. pombe*. Therefore, the majority of centromeric sequences in *S. pombe* have the potential to initiate replication. This activity in replication might indicate that more potential initiation sites are required in centromeric DNA than in bulk DNA, perhaps because the centromeric DNA, as a result of altered conformation and possible condensation, may be less amenable to replication initiation. Alternatively, the prevalence of ARS elements in the centromere might indicate that at least one DNA-binding protein involved in replication is also involved in centromere function. This idea can be tested when centromeric and replication proteins in *S. pombe* are identified.

ACKNOWLEDGMENTS

J.G.S. was partially supported by Cancer Biology training grant PHS NRSA CA 09302-16. G.H.B. was supported by training grant EY-07108 from the National Institutes of Health. This work was supported by grants from the National Institute of General Medical Science (GM33783) to L.C. and the American Cancer Society (VM-123) to M.P.C.

REFERENCES

1. **Baum, M., V. K. Ngan, and L. Clarke.** 1994. The centromeric K-type repeat and the central core are together sufficient to establish a functional *Schizosaccharomyces pombe* centromere. Mol. Biol. Cell **5:**747–761.
2. **Brewer, B. J., and W. L. Fangman.** 1987. The localization of replication origins on ARS plasmids in *S. cerevisiae*. Cell **51:**463–471.
3. **Brewer, B. J., and W. L. Fangman.** 1994. Initiation preference at a yeast origin of replication. Proc. Natl. Acad. Sci. USA **91:**3418–3422.
4. **Brewer, B. J., D. Lockshon, and W. L. Fangman.** 1992. The arrest of replication forks in the rDNA of yeast occurs independently of transcription. Cell **71:**267–276.
5. **Caddle, M. S., and M. P. Calos.** 1992. Analysis of the autonomous replication behavior in human cells of the dihydrofolate reductase putative chromosomal origin of replication. Nucleic Acids Res. **20:**5971–5978.
6. **Caddle, M. S., and M. P. Calos.** 1994. Specific initiation at an origin of replication from *Schizosaccharomyces pombe*. Mol. Cell. Biol. **14:**1796–1805.
7. **Campbell, J. L.** 1986. Eukaryotic DNA replication: yeast bares its ARSs. Annu. Rev. Biochem. **55:**733–771.
8. **Campbell, J. L., and C. S. Newlon.** 1991. Chromosomal DNA replication, p. 41-146. *In* J. R. Broach, J. R. Pringle, and E. W. Jones (ed.), The molecular biology and cellular biology of the yeast *Saccharomyces*: genome dynamics, protein synthesis, and energetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

9. **Chikashige, Y., N. Kinoshita, Y. Nakaseko, T. Matsumoto, S. Murakami, O. Niwa, and M. Yanagida.** 1989. Composite motifs and repeat symmetry in *S. pombe* centromeres: direct analysis by integration of *Not*I restriction sites. Cell **57:**739–751.
10. **Clarke, L., M. Baum, L. G. Marschall, V. K. Ngan, and N. C. Steiner.** 1993. Structure and function of *Schizosaccharomyces pombe* centromeres. Cold Spring Harbor Symp. Quant. Biol. **58:**687–695.
11. **Clarke, L., and M. P. Baum.** 1990. Functional analysis of a centromere from fission yeast: a role for centromere-specific repeated DNA sequences. Mol. Cell. Biol. **10:**1863–1872.
12. **Clarke, L., and J. Carbon.** 1985. The structure and function of yeast centromeres. Annu. Rev. Genet. **19:**29–56.
13. **Cottarel, G., J. H. Shero, P. Hieter, and J. H. Hegemann.** 1989. A 125-base-pair *CEN6* DNA fragment is sufficient for complete meiotic and mitotic centromere functions in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**3342–3349.
14. **Dershowitz, A., and C. S. Newlon.** 1993. The effect on chromosome stability of deleting replication origins. Mol. Cell. Biol. **13:**391–398.
15. **Deshpande, A. M., and C. S. Newlon.** 1992. The ARS consensus sequence is required for chromosomal origin function in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:**4305–4313.
16. **Dubey, D. D., L. R. Davis, S. A. Greenfeder, L. Y. Ong, J. Zhu, J. R. Broach, C. S. Newlon, and J. A. Huberman.** 1991. Evidence suggesting that the ARS elements associated with silencers of the yeast mating-type locus *HML* do not function as chromosomal origins of replication. Mol. Cell. Biol. **11:**5346–5355.
17. **Dubey, D. D., J. Zhu, D. L. Carlson, K. Sharma, and J. A. Huberman.** 1994. Three ARS elements contribute to the *ura4* replication origin region in the fission yeast, *Schizosaccharomyces pombe*. EMBO J. **13:**3638–3647.
18. **Fishel, B., H. Amstutz, M. Baum, J. Carbon, and L. Clarke.** 1988. Structural organization and functional analysis of centromeric DNA in the fission yeast *Schizosaccharomyces pombe*. Mol. Cell. Biol. **8:**754–763.
19. **Greenfeder, S. A., and C. S. Newlon.** 1992. A replication map of a 61-kb circular derivative of *Saccharomyces cerevisiae* chromosome III. Mol. Biol. Cell **3:**999–1013.
20. **Hahnenberger, K. M., J. Carbon, and L. Clarke.** 1991. Identification of DNA regions required for mitotic and meiotic functions within the centromere of *Schizosaccharomyces pombe* chromosome I. Mol. Cell. Biol. **11:**2206–2215.
21. **Heinzel, S. S., P. J. Krysan, C. T. Tran, and M. P. Calos.** 1991. Autonomous DNA replication in human cells is affected by the size and source of the DNA. Mol. Cell. Biol. **11:**2263–2271.
22. **Hsiao, C., and J. Carbon.** 1979. High frequency transformation of yeast by plasmids containing the cloned yeast *ARG4* gene. Proc. Natl. Acad. Sci. USA **76:**3829–3833.
23. **Huberman, J. A., L. D. Spotila, K. A. Nawotka, S. M. El-Assouli, and L. R. Davis.** 1987. The *in vivo* replication origin of yeast 2μm plasmid. Cell **51:**473–481.
24. **Krysan, P. J., and M. P. Calos.** 1991. Replication initiates at multiple locations on an autonomously replicating plasmid in human cells. Mol. Cell. Biol. **11:**1464–1472.
25. **Krysan, P. J., S. B. Haase, and M. P. Calos.** 1989. Isolation of human sequences that replicate autonomously in human cells. Mol. Cell. Biol. **9:**1026–1033.
26. **Krysan, P. J., J. G. Smith, and M. P. Calos.** 1993. Autonomous replication in human cells of multimers of specific human and bacterial DNA sequences. Mol. Cell. Biol. **13:**2688–2696.
27. **Linskens, M. H. K., and J. A. Huberman.** 1988. Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8:**4927–4935.
28. **Marschall, L. G., and L. Clarke.** 1995. A novel cis-acting centromeric DNA element affects *S. pombe* centromeric chromatin structure at a distance. J. Cell Biol. **128:**445–454.
29. **Maundrell, K., A. Hutchinson, and S. Shall.** 1988. Sequence analysis of ARS elements in fission yeast. EMBO J. **7:**2203–2209.
30. **Maundrell, K., A. P. H. Wright, M. Piper, and S. Shall.** 1985. Evaluation of heterologous ARS activity in *S. cerevisiae* using cloned DNA from *S. pombe*. Nucleic Acids Res. **13:**3711–3722.
31. **Moreno, S., A. Klar, and P. Nurse.** 1991. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. **194:**795–823.
32. **Murakami, S., T. Matsumoto, O. Niwa, and M. Yanagida.** 1991. Structure of the fission yeast centromere *cen3*: direct analysis of the reiterated inverted region. Chromosoma **101:**214–221.
33. **Nakaseko, Y., Y. Adachi, S. Funahashi, O. Niwa, and M. Yanagida.** 1986. Chromosome walking shows a highly homologous repetitive sequence present in all the centromere regions of fission yeast. EMBO J. **5:**1011–1021.
34. **Newlon, C. S.** 1988. Yeast chromosome replication and segregation. Microbiol. Rev. **52:**568–601.
35. **Newlon, C. S., L. R. Lipchitz, I. Collins, A. Deshpande, R. J. Devenish, R. P. Green, H. L. Klein, P. T. G., R. Ren, S. Synn, and S. T. Woody.** 1991. Analysis of a circular derivative of *Saccharomyces cerevisiae* chromosome III: a physical map and identification and location of *ARS* elements. Genetics **129:**343–357.
36. **Newlon, C. S., and J. F. Theis.** 1993. The structure and function of yeast ARS elements. Curr. Opin. Dev. Genet. **3:**752–758.
37. **Polizzi, C., and L. Clarke.** 1991. The chromatin structure of centromeres from fission yeast: differentiation of the central core that correlates with function. J. Cell Biol. **112:**191–201.
38. **Prentice, H. L.** 1991. High efficiency transformation of *Schizosaccharomyces pombe* by electroporation. Nucleic Acids Res. **20:**621.
39. **Smith, J. G., and M. P. Calos.** Autonomous replication in *Drosophila melanogaster* tissue culture cells. Chromosoma, in press.
40. **Steiner, N. C., and L. Clarke.** 1994. A novel epigenetic effect can alter centromere function in fission yeast. Cell **79:**865–874.
41. **Steiner, N. C., K. M. Hahnenberger, and L. Clarke.** 1993. Centromeres of the fission yeast *Schizosaccharomyces pombe* are highly variable genetic loci. Mol. Cell. Biol. **13:**4578–4587.
42. **Stinchcomb, D. T., K. Struhl, and R. W. Davis.** 1979. Isolation and characterisation of a yeast chromosomal replicator. Nature (London) **282:**39–43.
43. **Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis.** 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA **76:**1035–1039.
44. **Takahashi, K., S. Murakami, Y. Chikashige, H. Funabiki, O. Niwa, and M. Yanagida.** 1992. A low copy number central sequence with strict symmetry and unusual chromatin structure in fission yeast centromere. Mol. Biol. Cell **3:**819–835.
45. **Van Bokkelen, G. B., and M. P. Calos.** Physical mapping of replication of a human centromere. Submitted for publication.
46. **Van Houten, J. V., and C. S. Newlon.** 1990. Mutational analysis of the consensus sequence of a replication origin from yeast chromosome III. Mol. Cell. Biol. **10:**3917–3925.
47. **Willard, H. F.** 1990. Centromeres of mammalian chromosomes. Trends Genet. **6:**410–416.
48. **Willard, H. F., and J. S. Waye.** 1987. Hierarchical order in chromosome-specific human alpha satellite DNA. Trends Genet. **3:**192–198.
49. **Williamson, D. H.** 1985. The yeast ARS element, six years on: a progress report. Yeast **1:**1–14.
50. **Wohlgemuth, J. G., G. H. Bulboaca, M. Moghadam, M. S. Caddle, and M. P. Calos.** 1994. Physical mapping of origins of replication in *Schizosaccharomyces pombe*. Mol. Biol. Cell **5:**839–849.
51. **Wright, A. P. H., K. Maundrell, and S. Shall.** 1986. Transformation of *Schizosaccharomyces pombe* by non-homologous, unstable integration of plasmids in the genome. Curr. Genet. **10:**503–508.
52. **Zhu, J., C. Brun, H. Kurooka, M. Yanagida, and J. A. Huberman.** 1992. Identification and characterization of a complex chromosomal replication origin in *Schizosaccharomyces pombe*. Chromosoma **102:**S7–S16.