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Isolation and Transcriptional Characterization of Three Genes Which Function at Start, the Controlling Event of the *Saccharomyces cerevisiae* Cell Division Cycle: *CDC36*, *CDC37*, and *CDC39*

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The genes *CDC36*, *CDC37*, and *CDC39*, thought to function in the cell division control process in *Saccharomyces cerevisiae*, were isolated from a recombinant plasmid library prepared by partial digestion of *S. cerevisiae* genomic DNA with *Sau*3A and insertion into the *S. cerevisiae-Escherichia coli* shuttle vector YRp7. In each case, *S. cerevisiae* DNA sequences were identified which could complement mutant alleles of the gene in question and which could direct integration of a plasmid at the chromosomal location known to correspond to that gene. Complementing DNA segments were subcloned to remove extraneous coding regions. The coding regions corresponding to *CDC36*, *CDC37*, and *CDC39* were then identified and localized by R-loop analysis. The estimated sizes of the three coding regions were 615, 1,400, and 2,700 base pairs, respectively. Transcriptional orientation of the coding regions was established by using M13 vectors to prepare strand-specific probes followed by hybridization to blots of electrophoresed *S. cerevisiae* mRNA. The intracellular steady-state abundance of the mRNA species corresponding to the genes was estimated by comparing hybridization signals on RNA blots to that of a previously determined standard, the cell cycle start gene *CDC28*. The quantities calculated for the three mRNA species were low, ranging from 1.5 ± 1 copies per haploid cell for the *CDC36* mRNA to 3.1 ± 1.5 and 4.6 ± 2 copies per haploid cell for the *CDC37* and *CDC39* mRNAs, respectively. The *CDC28* mRNA had been previously estimated at 7.0 ± 2 copies per cell.

Cell division is a fundamental process of living organisms. Owing to its complexity, however, little has been learned about the molecular events which constitute it or control it. In *Saccharomyces cerevisiae*, it has been possible through mutational analysis to identify genetic loci specific for cell division and its control (10, 11, 27, 28). The isolation and characterization of mutations which interrupt the cell division cycle at specific stages (*cdc* mutations) has led to the formulation of a model of the cell cycle as a set of several interlocking subcycles, each organized in a dependent sequence of events which require the expression of specific genes (9, 10, 14, 27). Because the various subcycles appear to converge during the G_1 interval of the cell cycle, and owing to the dependent interrelationships of the events contained within each subcycle, control of cell division can, in principle, be effected by regulating a single G_1 event. This event has been given the operational designation of "start" (4, 5, 10, 12, 18, 27). It has been observed that cell division in *S. cerevisiae* cells responding either to nutrient limitation or to mating pheromone as a prelude to conjugation, stops at the same point in the G_1 segment of the cell cycle, consistent with the "single control point" hypothesis. Therefore, in the interest of investigating cell cycle control, mutants conditionally incapable of executing the start step were sought. Forty start mutants which mimic pheromonally arrested cells were isolated and assigned to four unlinked complementation groups: *CDC28*, *CDC36*, *CDC37*, and *CDC39* (28).

One approach to understanding gene function, made feasible by the advent of recombinant DNA methodologies, is the physical isolation of genes. In *S. cerevisiae*, this can be readily achieved by transformational complementation (1, 17). Once genes have been isolated, strate-

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gies for enlisting them as reagents for the investigation of both the transcriptional and translational products they encode may be employed. We have recently reported the isolation of one of the start genes, *CDC28* (24), the preliminary description of its transcriptional and translational products (30), and finally the use of the cloned gene to prepare antisera specific for the *CDC28* gene product (29). We intend to extend similar studies to the remaining three start genes, *CDC36*, *CDC37*, and *CDC39*. To that end, we report here the isolation and characterization of the transcriptional coding regions for these three genes.

MATERIALS AND METHODS

Bacterial and *S. cerevisiae* strains and culture media. *Escherichia coli* strains sf8 (C600, *lop-11 hsdM hsdR recBC*) and JA300 (C600, *trpC hsdM hsdR recBC*) were used except for M13 cloning, for which JM103 [C600, Δ(*lac pro*) *endA sbcB hsdR* F′ *traD proAB lacI*^{q} ZΔM15] was used. The *S. cerevisiae* strains used in this study and their genotypes are given in Table 1. Yeast and bacterial culture media used have been described previously (6, 8).

Plasmids and cloning procedures. The source of complementing yeast sequences was a library prepared by partial digestion of *S. cerevisiae* DNA with *Sau*3A and insertion into the shuttle vector YRp7 (24). Subclone libraries in vectors pRC1, pRC2, and pRC3 were as has been described (6). *E. coli* and *S. cerevisiae* transformations, as well as other cloning procedures, have been described in previous reports (6, 29). M13 cloning procedures using M13mp7, -mp8, and -mp9 were performed essentially as is described in the Bethesda Research Laboratories M13 cloning manual. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs or Bethesda Research Laboratories or were prepared in the undergraduate Biochemistry Laboratory Course (Bio 109L) at the University of California, Santa Barbara, and were used according to the manufacturers' specifications. Double-strand nuclease *Bal*31 was purchased from Bethesda Research Laboratories and used as suggested. It was necessary to precalibrate the enzyme for each DNA substrate used, so as to generate deletions of the desired extent.

***S. cerevisiae* genetics.** Two procedures were used to prepare *S. cerevisiae* strains containing integrated plasmids. For experiments to confirm the identity of plasmids capable of complementing *cdc36* mutations, *S. cerevisiae* TP302-1, transformed with YRp7(*CDC36*.1), an autonomously replicating plasmid, was passaged serially in nonselective medium for 20 to 30 generations. Under these conditions, only the subset of the population containing an integrated plasmid would be expected to maintain the TRP$^+$ phenotype (capability of growth on tryptophanless medium). Such putative integrant strains were then identified by plating on tryptophanless medium. TRP$^+$ clones were tested for mitotic stability by repassaging nonselectively and then by replica plating to confirm the stable TRP$^+$ phenotype. For *CDC37* and *CDC39*, plasmid DNA containing *S. cerevisiae* sequences but not an autonomous replication segment was linearized by restriction endonuclease cleavage at a unique site within the yeast insert to direct integration into homologous chromosomal sequences (26). Presumptive integrant transformants were then crossed to appropriately marked tester strains. Tetrad analysis (22) was performed to map the point of integration as has been described (24).

TABLE 1. *S. cerevisiae* strains

Strain	*cdc* gene	Genotype
TP301-1	*CDC36*	*cdc36-16 MAT*α *trp1 ura1 ilv2*
TP302-1	WT[a]	*MAT***a** *ste7 trp1 met8 ile1 ade2*
SR664-1	WT[b]	*MAT***a** *trp1 hom2 met8 aro1 arg4 cdc15*
JF214-2	*CDC37*	*cdc37-1 MAT*α *trp1 met2*
JF212-1	*CDC39*	*cdc39-1 MAT*α *trp1 met2 ura1 his7*
JF212-9	*CDC39*	*cdc39-1 MAT***a** *trp1 met2*
RH218	WT	*trp1*

[a] WT, Wild type.
[b] This strain carries a *cdc* mutation, *cdc15-1*, which is irrelevant to this study.

R-loop analysis. Homologous RNA was purified and R-loops were prepared as has been described (30, 35), except that the R-loops were fixed by addition of glyoxal to 1 M at 12°C for 2 h (19) before preparation for electron microscopic observation. Electron microscopy with a Siemens Elmskop 1 microscope and treatment of data were carried out as previously described (30).

RNA transfer procedures. RNA electrophoresis and transfer were carried out essentially as described by Thomas (36), except that glyoxal gels were run in 40 mM morpholinepropanesulfonic acid buffer (pH 7) rather than 10 mM sodium phosphate. Blots were hybridized to nick-translated probes (31) in the presence of 10% dextran sulfate as has been described (30). For transcriptional orientation experiments, unlabeled single-strand DNA probes derived from M13 clones were hybridized (0.05 μg per nitrocellulose strip) for 12 to 16 h, followed by extensive washing as has been described (30). The blots were then reacted with nick-translated (31) M13mp7 replicative form DNA for 4 h, followed again by extensive washing. *E. coli* DNA polymerase holoenzyme for nick translation was purchased from Boehringer Mannheim Corp. [α-^{32}P]dTTP was obtained from Amersham Corp. Plasmids and double-strand phage DNAs were labeled to a specific activity of 10^8 cpm/μg. Hybridized blots were exposed to Kodak XAR-5 film at −75°C in the presence of a Cronex Lightning-Plus intensifying screen (Du Pont Co.). Densitometry was performed by using a E-C 810 densitometer and by weighing excised peaks on a Mettler balance. Data analysis was as has been described previously (30).

RESULTS

Isolation of plasmids capable of complementing *cdc36*, *cdc37*, and *cdc39* mutations. The identification of cell cycle start mutants has been de-

scribed (28). Strains carrying the alleles *cdc36-16*, *cdc37-1*, and *cdc39-1* were constructed to contain the *trp1-1* mutation as well. A library produced by partial digestion of *S. cerevisiae* DNA by *Sau*3A and insertion into the *Bam*HI site of YRp7 (24) served in each case as the source of complementing sequences. Plasmid DNA was isolated from TRP^+ CDC^+ transformant yeast colonies and used to transform *E. coli* for amplification and analysis. For each mutant, several plasmids containing inserts of different sizes and structures were obtained which could be demonstrated to possess complementing activity. Preliminary restriction analysis of each set usually suggested sequence homology between members, on the basis of fragments of common electrophoretic mobility; however, this was not always the case (data not shown). Rather than invest a great deal of effort on the analysis of primary clones which were expected to contain at least several transcribed sequences (13, 16, 25, 33), we chose a strategy of subcloning the sequences of interest before detailed analysis. In each case, the plasmid containing the smallest complementing insert was used as the source of DNA for subcloning.

Subcloning the start genes. Two strategies for subcloning were employed. The first entailed producing a sublibrary of the primary clone by redigestion with *Sau*3A under conditions calibrated to give relatively small fragments, followed by insertion into a new vector system. Recombinant plasmids were then again selected on the basis of their being able to complement the start mutation upon transformation. This approach and the vectors used are described extensively elsewhere (6).

An alternative strategy was to subclone specific restriction fragments isolated by polyacrylamide gel electrophoresis (29) or to subclone by removal of convenient restriction fragments from preexisting plasmids. This approach was particularly attractive for *CDC36*, because two primary clones isolated, YRp7(*CDC36*.1) and YRp7(*CDC36*.2), were found to have a very restricted region of overlap (Fig. 1). Two subclones were especially useful in localizing the *CDC36*-coding region. pRC2(*CDC36*.2) was constructed by isolating a *Sac*I fragment from YRp7(*CDC36*.1) (Fig. 1) and inserting it into the unique *Sac*I site of pRC2. YRp7(*CDC36*.3) was constructed by removal from YRp7(*CDC36*.2) of an *Sph*I fragment extending from a site in the vector to one near the left end of the yeast insert (Fig. 1). Most of the insert is thus deleted, leaving only 1 kilobase pair (kbp). Both of these subclones were capable of complementing *cdc36* mutations. The *Sau*3A partial digest method was also used, yielding pRC2(*CDC36*.1) which has an insert similar to that in pRC2(*CDC36*.2).

The primary clone capable of complementing *cdc37* mutations which contained the smallest

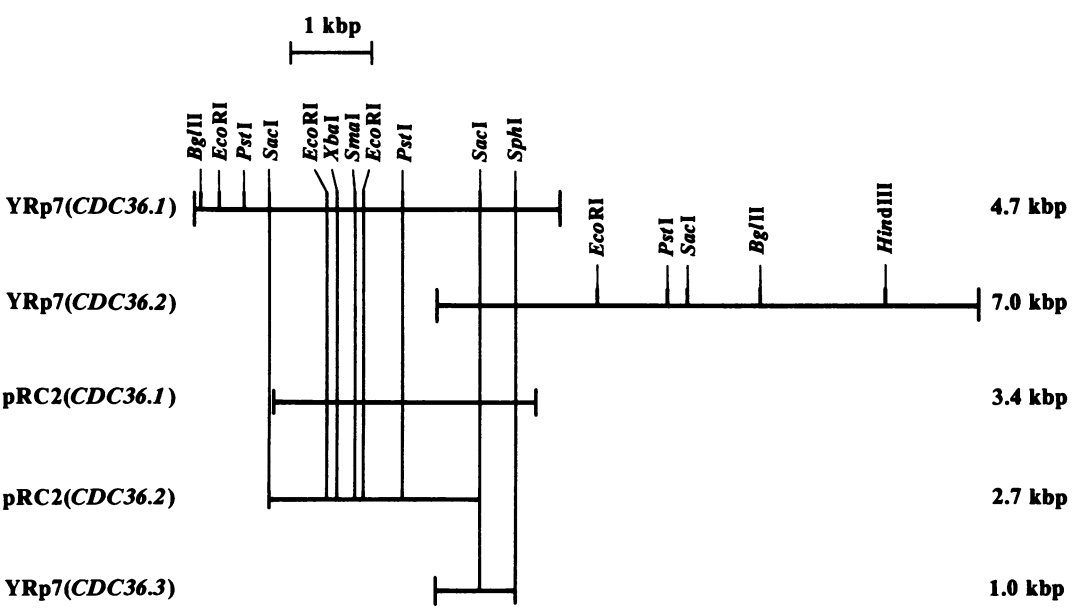

FIG. 1. Plasmid inserts containing *CDC36* sequences. The inserted *CDC36*-containing *S. cerevisiae* sequences were isolated from a library of yeast genomic fragments (24), and subsequent subclones were aligned. Representative restriction sites are shown. All of the DNA segments depicted are capable of complementing the *cdc36-16* temperature-sensitive mutation.

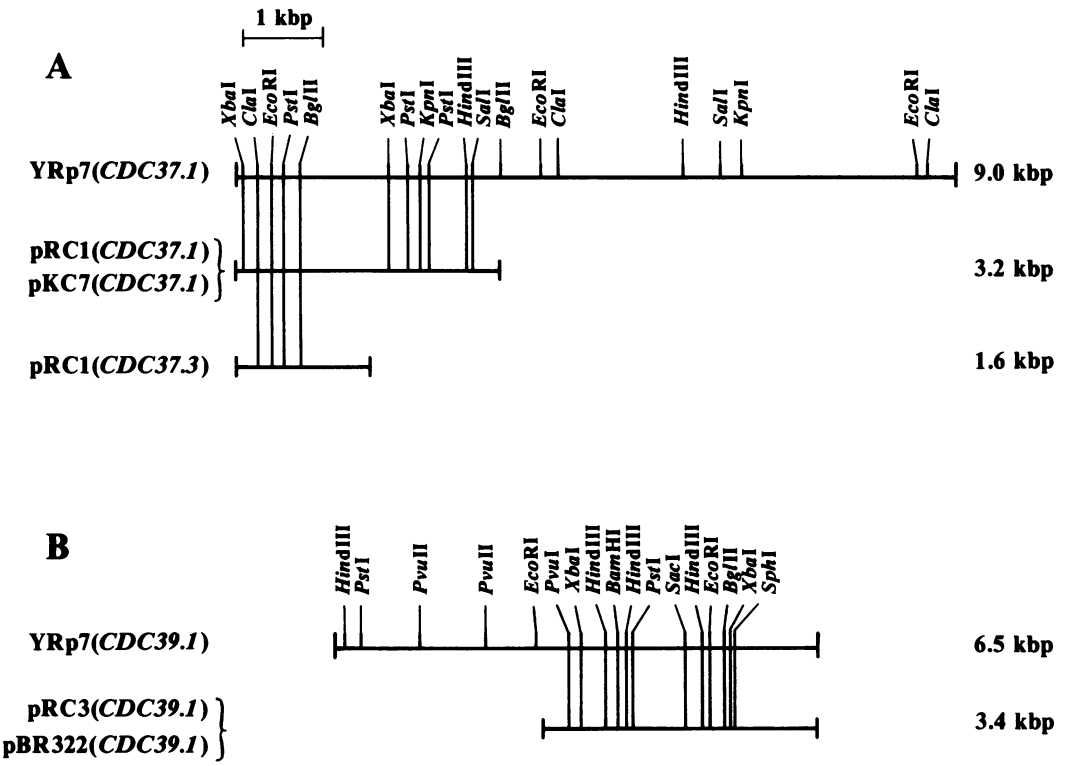

FIG. 2. Plasmid inserts containing *CDC37* and *CDC39* sequences. The inserted *CDC37*-containing (A) and *CDC39*-containing (B) sequences isolated from a library of yeast genomic fragments (24) and subsequent subclones are aligned with representative restriction sites indicated. In the case of *CDC39*, restriction site assignments in the portion of YRp7(*CDC39*.1) not included in pRC3(*CDC39*.1) are tentative, as a detailed restriction analysis was not performed before subcloning.

yeast DNA insert (9.0 kbp), YRp7(*CDC37*.1), was subcloned into pRC1 by the *Sau*3A partial digestion method. Of several subclones still capable of complementing the *cdc37-1* mutation, that containing the smallest yeast insert again, pRC1(*CDC37*.1), was chosen for further study. A restriction map of the 3.2-kbp insert is shown in Fig. 2A. This subclone was found to have the same left endpoint as the parental clone, YRp7(*CDC37*.1).

CDC39 was subcloned in an analogous fashion except that the vector pRC3 was used. The primary clone, YRp7(*CDC39*.1), which contained an insert of 6.0 kbp, yielded a subclone, pRC3(*CDC39*.1), containing an insert of 3.4 kbp (Fig. 2B). It was determined, based on Southern blots, that a small segment at the left end of this insert was derived from vector rather than yeast sequences (data not shown). Subsequent DNA sequence analysis has confirmed the presence of a 78-base pair (bp) *Sau*3A fragment derived from the pBR322 portion of the parental clone (unpublished data). The intrusion of such noncontiguous segments is a potential artifact associated with this subcloning procedure. The right endpoints of the parental clone and the subclone are identical.

Confirmation of identity of the cloned sequences. The identity of the cloned sequences was confirmed by demonstration of their ability to integrate, presumably by homologous recombination, at the appropriate chromosomal loci. This method was used previously in the analysis of DNA segments capable of complementing *cdc28* mutations (24). For some of these studies, we enhanced the efficiency of integration at the homologous chromosomal site by removing the *S. cerevisiae* segment which confers the ability to replicate autonomously (*ars*) and by linearizing the plasmid by cleavage at a unique restriction site within the yeast insert segment. This has been shown to direct integration specifically at the point defined by the ends of the linearized plasmid (26). In each case, then, the *TRP1* marker associated with the vector was used to map the position of integration with respect to

the *S. cerevisiae* genetic map. The data derived from tetrad analysis performed on crosses of integrant strains are summarized in Table 2. In each instance, the vector-associated *TRP1* marker was found to be tightly linked to the expected chromosomal markers, confirming the identity of the gene isolated.

Mapping of transcriptional coding regions on the cloned segments. The organization of transcriptional units within the cloned segments was determined by R-loop analysis as has been described previously (30, 35). RNA species complementary to the DNA to be analyzed were purified from polyadenylate-enriched RNA by hybridization to DNA immobilized on diazobenzyloxymethyl-paper (7). Such prior enrichment of RNA species greatly facilitated their analysis, as they were all of low intracellular abundance (see below). An additional modification of the standard R-loop preparation procedure that proved to be helpful was fixation of the R-loops with glyoxal before preparation for electron microscopy (19). This procedure improved the recovery of R-loop–containing molecules in terms both of absolute frequencies of such molecules observed and of reproducibility of R-loop frequency from experiment to experiment. There is also evidence that this procedure, by eliminating strand displacement at R-loop ends, results in a more accurate determination of the

TABLE 2. Linkage of integrated (int) plasmids

Cross	No. of asci	Loci scored	Ascus type[a]			
			PD	NPD	T	Other[a]
TP301-1 × TP302-1 int YRp7(*CDC36*.1)	37	*TRP1*[b], *ste7*[c]	28	0	2	7
SR664-1 × JF214-2 int1 pRC1′(*CDC37*.4)[d]	7	*TRP1*, *hom2*[e]	7	0	0	0
SR664-1 × JF214-2 int2 pRC1′(*CDC37*.4)	18	*TRP1*, *hom2*	17	0	0	1
JF212-9 × JF212-1 int1 YRp7′(*CDC39*.2)[f]	15	*TRP1*, *CDC39*[g]	15	0	0	0
RH218 × JF212-1 int1 YRp7(*CDC39*.2)	14	*CDC39*, *cdc39*[h]	14	0	0	0

[a] "Other" includes asci for which 2:2 segregation was not observed for both markers scored. All such instances could be explained on the basis of gene conversion or plasmid loss. PD, Parental ditype; NPD, nonparental ditype; T, tetratype.

[b] In each analysis, *TRP1* is a plasmid-associated marker and identifies the point of plasmid integration. It should not be confused with the genomic *trp1* locus, which remains silent in these experiments.

[c] *ste7* has been shown to be 2 meiotic map units from *cdc36* (23; our unpublished data).

[d] pRC1′(*CDC37*.4) was constructed as follows. In pRC1(*CDC37*.1) the insert is oriented with its unique *Eco*RI site proximal to the kanamycin resistance (Km^r) locus. After cleavage of pRC1(*CDC37*.1) with *Eco*RI and religation, screening was performed to obtain pRC1′(*CDC37*.2). This plasmid was deleted for the *Eco*RI fragment containing the Km^r locus and a portion of the *CDC37* coding region (see Fig. 4A) and had the *Eco*RI fragment containing the *TRP1* and *ars1* segments now in the inverted orientation. Cleavage of this plasmid with *Bgl*II, followed by dilution and religation, gave pRC1′(*CDC37*.4), which retains *TRP1* function but is deleted for *ars* function and an additional portion of the *CDC37* coding region. This plasmid was linearized by cleavage with *Kpn*I and transformed to JF214-2 to give stable TRP^+ strains presumably by integration.

[e] *hom2* has been shown to be 2 meiotic map units from *cdc37* (28).

[f] YRp7′(*CDC39*.2) was constructed in the following manner. pRC3(*CDC39*.1) was partially digested with *Eco*RI, religated, and transformed to *E. coli*. Kanamycin-sensitive colonies were screened, yielding pBR322(*CDC39*.1), from which the *TRP1 ars1* and Km^r-bearing *Eco*RI fragments had been deleted while the *Eco*RI site within the *S. cerevisiae* insert segment remained intact. As a fortuitous result of the *Sau*3A subcloning procedure used to construct pRC3(*CDC39*.1), pBR322(*CDC39*.1) has a novel *Bam*HI site at each vector-insert junction, as well as an additional site within the insert. pBR322(*CDC39*.1) was then partially digested with *Bam*HI and ligated with a *Bam*HI-*Bgl*II digest of YRp7′. YRp7′ is analogous to YRp7 (34) except that the *Eco*RI fragment containing *TRP1* and *ars1* function is inverted with respect to pBR322. Recombinant plasmids were screened for transfer of the entire 3.4-kb *CDC39*-complementing segment to replace the smaller vector segment originally spanning the *Bgl*II and *Bam*HI sites. Such a construction is feasible because *Bgl*II and *Bam*HI produce identical cohesive ends. YRp7′(*CDC39*.2) therefore has intact *CDC39*- and *TRP1*-coding regions but is deleted for *ars1* function. This plasmid was linearized by cleavage with *Sac*I and transformed to JF212-1 to give stable CDC^+ TRP^+ strains, presumably by integration.

[g] This cross indicates that the CDC^+ and TRP^+ phenotypes are meiotically stable and linked, presumably because they are both contained on the same integrated plasmid.

[h] The linkage of the plasmid-associated *CDC39* allele to the genomic *cdc39* allele is demonstrated by the absence of recombinant tetrads. The latter would be identified by the appearance of temperature-sensitive spores. None was observed.

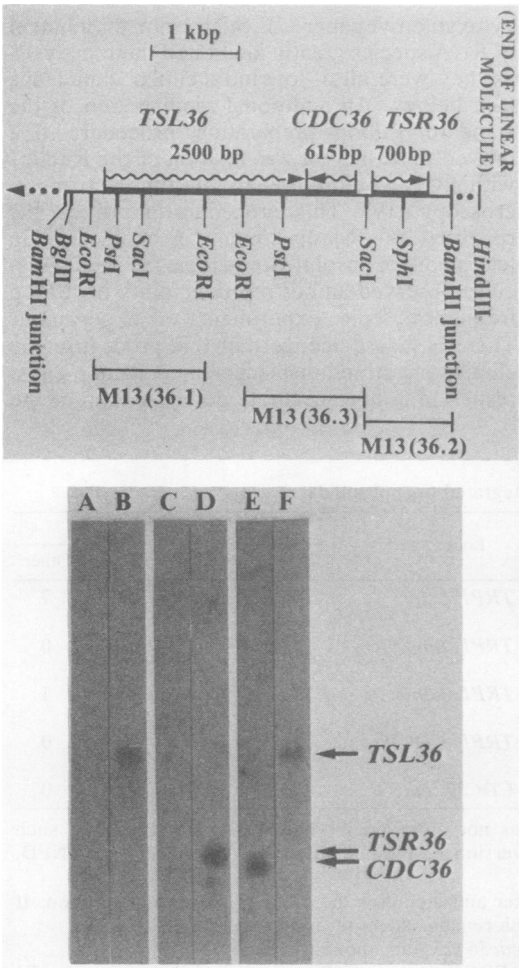

FIG. 3. RNA coding regions included in YRp7(*CDC36*.1) and their transcriptional orientation. Collection and treatment of R-loop data were performed as has been described (30). Coding regions were positioned and sized based on 17 R-loop–containing molecules corresponding to *TSR36*, 30 R-loop–containing molecules corresponding to *CDC36*, and 22 R-loop–containing molecules corresponding to *TSL36*. Transcriptional orientation was determined with M13 cloning systems (19) as described in the text. Samples run in lanes A and B were probed with single strands of the *Eco*RI fragment, designated M13(36.1). Appropriate M13 clones were prepared by inserting this fragment into the *Eco*RI cloning site of M13mp7 in the two possible orientations. These probes have homology with only the *TSL36* coding region. Samples in lanes C and D were probed with single strands of the *Bam*HI-*Sac*I fragment, designated M13(36.2), which was cloned by trimming ends with *Bal*31 and inserting the blunt-ended fragment into the *Hin*cII site of M13mp7 in the two possible orientations. These probes have homology with only the *TSR36* coding region. Samples in lanes E and F were probed by using single strands of the *Eco*RI-*Sac*I fragment, designated M13(36.3). These clones were obtained by inserting a larger *Eco*RI-*Bam*HI fragment extending to the insert-coding region under analysis (19; our unpublished data).

YRp7(*CDC36*.1) was linearized for R-loop analysis by cleavage with *Hin*dIII. The plasmid is converted into two linear fragments, the larger of which contains the insert sequences and most of the vector. The smaller segment (ca. 600 bp) is composed entirely of vector sequences, including most of the *TRP1* coding region. The *S. cerevisiae* insert of YRp7(*CDC36*.1) was found to contain at least three RNA coding regions (Fig. 3). The topologies of subclones pRC2(*CDC36*.1), pRC2(*CDC36*.2), and YRp7(*CDC36*.3) allowed the establishment of the central coding region as *CDC36*. Based on R-loop analysis, which excludes the contribution of polyadenylation, the *CDC36* transcript contains slightly over 600 nucleotides.

pRC1(*CDC37*.1) was modified for use in the R-loop analysis of *CDC37*. The segment of the vector that contained the *TRP1* coding region was removed so it would not interfere with subsequent analysis by forming irrelevant R-loops. The *TRP1 ars1* segment of the vector was deleted by partial digestion with *Eco*RI followed by religation. An *Eco*RI site within the yeast insert region of the plasmid necessitated this strategy. Screening was then required to obtain a plasmid which had retained the complete *CDC37*-complementing insert but had lost the 1.4-kb *Eco*RI fragment containing the *TRP1* coding region. This plasmid, pKC7(*CDC37*.1), was linearized by using a unique *Sma*I site in the vector region and reacted with prepurified complementary RNA. Two coding regions were identified (Fig. 4A). One appeared to be deleted of approximately 200 bp based on the existence of a single-strand tail of this size on the left end of virtually every R-loop of that class (Fig. 4A). Although this result implied, owing to the incomplete status of this coding region, that the other coding region detected (*TSR37* [transcribed sequence to the right of *CDC37*] in Fig. 4A) must correspond to *CDC37*, it was thought prudent to test this hypothesis by further deletion analysis. We used partial digestion of YRp7(*CDC37*.1) with *Eco*RI to construct a plasmid in which the vector *TRP1 ars1* segment was

vector junction of YRp7(*CDC36*.1) into both M13mp8 and M13mp9. A deletion was then prepared in each of these phages by digesting RF DNA with *Sac*I and *Bam*HI, trimming ends with *Bal*31, and religating. Lanes E and F correspond to the M13mp8 and M13mp9 derivatives, respectively. These probes have homology with both the *CDC36* and *TSL36* coding regions. The probes used in lanes A, C, and E contain insert sequences in the same orientation with respect to the vector sequences.

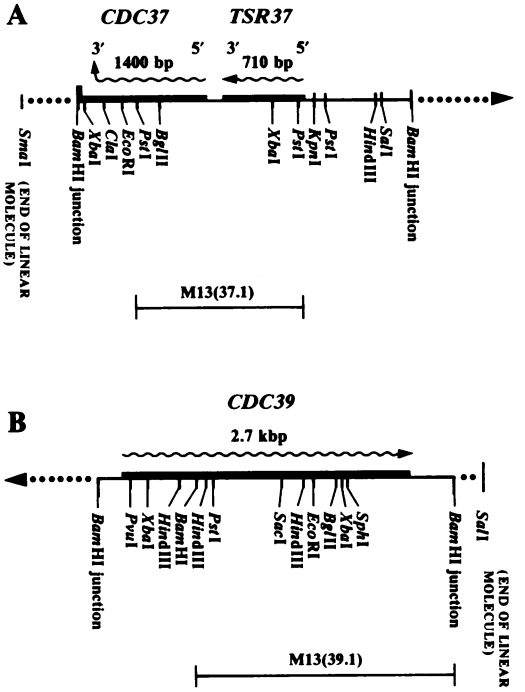

FIG. 4. RNA coding regions included in pRC1(*CDC37*.1) and pRC3(*CDC39*.1) and their transcriptional orientations. Collection and treatment of R-loop data were performed as has been described (30). Coding regions were positioned and sized based on 32 R-loop–containing molecules for *TSR37*, 18 R-loop–containing molecules for *CDC37* (A), and 62 R-loop–containing molecules for *CDC39* (B). Transcriptional orientation was established for *CDC37* and *TSR37* by using the *Pst*I fragment, designated M13(37.1), cloned into M13mp7 in the two possible orientations (A). The experiment was carried out analogously to that shown in Fig. 3. Transcriptional orientation was established for *CDC39* by using the *Bam*HI fragment, designated M13(39.1), cloned into M13mp7 in the two possible orientations (B). In this experiment, a second convergently transcribed coding region was detected (not shown because its precise position is not known). Only a portion of this coding region is included in the pRC3(*CDC39*.1) insert.

retained but a second segment, containing part of the left-hand coding sequence in Fig. 4A (leftward of the *Eco*RI site) and the vector region encoding kanamycin resistance, was eliminated. Surprisingly, this plasmid, pRC1(*CDC37*.2), was found to be incapable of complementing the *cdc37-1* mutation. A second deletion was prepared by digestion of pRC1(*CDC37*.1), linearized at the unique *Kpn*I site, with the double-strand nuclease *Bal*31. pRC1(*CDC37*.3) contains a deletion of approximately 2 kbp, eliminating most of the right-hand coding region shown in Fig. 4A. This plasmid was capable of complementing the *cdc37-1* mutation. In this manner, the leftward coding region, 1,400 bp in length, was established as the *CDC37* gene, even though in several complementing plasmids a small segment (200 bp) complementary to the mRNA was found to be deleted. The possible implications of this will be discussed below.

pRC3(*CDC39*.1) was modified for R-loop analysis in a fashion similar to that described for pRC1(*CDC37*.1). Partial digestion with *Eco*RI was used to remove vector sequences extraneous to the analysis while leaving the single *Eco*RI site in the *CDC39*-complementing yeast insert intact. The resulting plasmid, pBR322(*CDC39*.1), was cleaved at the unique *Sal*I site in the vector region and used in the R-loop analysis of hybrid-selected RNA. Only one coding region of 2.7 kbp was found in the 3.4-kbp insert. This was then assumed to correspond to the *CDC39* gene.

Determination of the transcriptional orientations of the start genes. The advent of cloning vectors derived from the bacteriophage M13 (21) provides a simple method for determining transcriptional orientation of a gene. The single plus strand of genomic DNA which is extruded from the host during infection by these vectors serves as a natural strand-specific probe. To determine the transcriptional orientation of a particular coding region, one need only prepare two derivatives containing the same DNA segment inserted in each of the possible orientations with respect to the phage genome. The plus-strand DNA, which can easily be prepared from infection supernatants, will then contain either the coding strand or the noncoding strand of the transcriptional unit under scrutiny, depending on the orientation of the cloned segment.

The application of M13 clones to the determination of the transcriptional orientation of coding regions on YRp7(*CDC36*.1), which contains *CDC36* and two additional genes, is demonstrated in Fig. 3. The segments cloned in both orientations into M13 vectors, designated M13(36.1), M13(36.2), and M13(36.3), are shown in relation to the transcriptional and restriction map of the region. Thus derivatives containing (36.1), (36.2), and (36.3) can orient *TSL36* (transcribed sequence to the left of *CDC36*), *TSR36*, and both *CDC36* and *TSL36*, respectively. The manner in which the experiment was carried out is illustrated in the bottom half of Fig. 3. Polyadenylate-enriched yeast RNA was separated on denaturing agarose gels and transferred to nitrocellulose membranes. Transfers from parallel lanes were cut into strips. Six of these were reacted separately with single-stranded DNA corresponding to the three segments indicated above in the two possible orientations. The

strips were then reacted with the nick-translated double-stranded replicative form (RF) of unrecombined vector to indicate the RNA-probe complementarities upon exposure to X-ray film. In Fig. 3, lanes A and B, C and D, and E and F correspond to derivatives of (36.1), (36.2), and (36.3), respectively. Lanes A, C, and E represent probe insert sequences in the same orientation with respect to the phage genome. The transcriptional scheme dictated by these results is indicated at the top of Fig. 3 by the arrows above each coding region. *CDC36* and *TSL36* are transcribed convergently, whereas *CDC36* and *TSR36* are transcribed divergently.

The transcriptional orientations of *CDC37* and the adjacent coding region *TSR37*, as well as that of *CDC39*, were determined in an analogous fashion (Fig. 4). Unlike the coding regions of the segment capable of complementing *cdc36* mutations, *CDC37* and *TSR37* are transcribed in parallel from the same DNA strand.

Quantitation of mRNAs by using RNA blots. We have reported previously a simple method for estimating intracellular abundance of an RNA species by comparison to a known standard (30). The method uses parameters obtainable from careful RNA blotting experiments in a hybridization-kinetics analysis format. Although assumptions must be made which are not rigorously testable, results have been obtained which were in good agreement with values derived by different procedures. In the experiments reported here, the *CDC28* transcript, quantitated previously at five to nine copies per haploid cell (30; Table 3), serves as the known standard. An example of a blot used in our analyses is shown in Fig. 5. Lanes A through C and D through F correspond to parallel transfers of serial RNA dilutions, separated on a 1.1% agarose gel (36) and probed with nick-translated M13(36.3) RF and pBR322(*CDC28*.1) DNA, respectively. Each probe has homology to two different RNA species. However, only those corresponding to *CDC36* and *CDC28* are included in the analysis here. Treatment of data and estimates of transcript abundance for *CDC36*, as well as for *CDC37* and *CDC39*, are shown in Table 3. None of the transcripts corresponding to these genes is abundant in a haploid cell. Both the *CDC37* and *CDC39* transcripts are present at approximately half the abundance of the *CDC28* transcript, whereas the *CDC36* transcript is maintained at one to two copies per haploid cell.

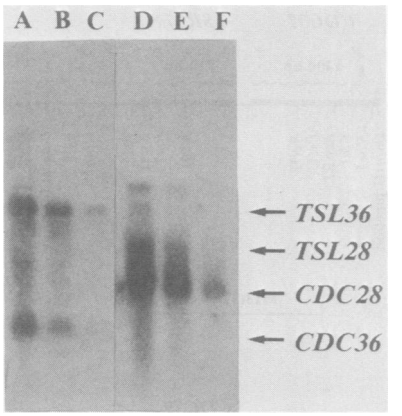

FIG. 5. RNA blots of *CDC36* and *CDC28* mRNAs. Oligodeoxythymidylate-cellulose-enriched *S. cerevisiae* RNA was electrophoresed on 1.1% agarose gels after denaturation by glyoxal and transferred to a nitrocellulose membrane by the method of Thomas (36). Parallel blots from the same gel were probed with nick-translated DNA from phage M13mp8(36.3), containing 615 bp of the *CDC36* coding sequence, or from plasmid pBR322(*CDC28*.1), containing 925 bp of the *CDC28* coding sequence. Each blot contains three lanes representing different amounts of RNA initially loaded on the agarose gel: (A) and (D), 15 μg; (B) and (E), 5 μg; (C) and (F), 1 μg.

DISCUSSION

We have reported here the isolation, subcloning, and preliminary characterization of three genes associated with control of cell division in *S. cerevisiae*. This study exemplifies a new type of approach to problems of cell biology which is made possible by the augmentation of genetic analysis with recombinant DNA technology. In essence, it is now reasonable to isolate physically any mutationally defined gene as a first step in clarifying how the gene's product is regulated and participates in cellular events. There are many different possible strategies for the pursuit of these objectives. All require, however, that the gene be located on the initially isolated segment of genomic DNA and separated from other extraneous coding sequences on this segment. In *S. cerevisiae*, this requirement is of special importance, because genes are spaced in the genome at a high density (13, 16, 25, 33). We have found in this study that the bulk of the DNA in segments analyzed codes for RNA, with only a small fraction characterizable as intergenic. With few exceptions, the most effective method of subcloning in our experience has been the selection of complementing fragments after redigestion of the original clones with a restriction endonuclease expected to have a large number of cleavage sites. We have described this method in detail elsewhere (6). Once smaller complementing segments have been isolated which are expected to contain no more than one or a few genes, transcriptional coding regions are localized by R-loop analysis. We have found that preselection of hybridizing RNA renders the method readily applicable for genes ex-

TABLE 3. Estimation of intracellular mRNA copy number[a]

Gene	Expt no.	Ratio of integrated peaks (I_{mRNA}/I_x)	Probe homology correction factor ($[H_x/H_{mRNA}]^{1.3}$)	Calculated mRNA copy no. (C_{mRNA})
CDC36[b]	1	0.076	1.70	0.65–1.16
	2	0.125	1.70	1.06–1.91
	3	0.172	1.70	1.46–2.63
	Avg			1.5 ± 1
CDC37[c]	1	0.376	1.00	1.88–3.38
	2	0.520	1.00	2.60–4.68
	Avg			3.1 ± 1.5
CDC39[d]	1	0.630	1.20	3.78–6.80
	2	0.470	1.20	2.82–5.08
	3	0.530	1.20	3.18–5.72
	Avg			4.6 ± 2

[a] In theory, the relative initial rates of two RNA-DNA hybridization reactions in solution can be described by the following relationship:

$$ir_x/ir_y = [(D_x/D_y)(H_x/H_y)] \times [(R_x/R_y)(H_x/H_y)]$$
$$= (D_x/D_y)(R_x/R_y)(H_x/H_y)^2$$

in which D and R are the concentrations of DNA and RNA species involved in the reaction, respectively, and H is the extent of homology between them, since the initial rate can only be a reflection of reaction between homologous sequences. For the purposes of this study, we shall assume that the extent of reaction as quantitated by film exposure and subsequent densitometry is a reflection of relative initial rates. The short hybridization times used and the relatively low specific radioactivity of bands on blots after hybridization support this interpretation. Since the same concentration of probe DNA went into each reaction, $D_x/D_y = 1$. R_x/R_y should equal the relative intracellular copy or abundance of the RNA species being probed, C_x/C_y. The exponent value for the homology factor of 2 results from the second-order nature of hybridization reactions in solution, where all potential duplex nucleation sites are free to diffuse. Assigning a proper exponent in this study is problematical, since only the probe nucleation sites are free to diffuse, whereas the RNA homologs are constrained to a degree that is difficult to assess. The effective order of the reaction, however, is expected to be reduced. An estimate for the order of the reaction has been determined empirically as 1.3 (30), giving the relationship:

$$I_x/I_y = (C_x/C_y)(H_x/H_y)^{1.3}$$

where I_x/I_y is the ratio of hybridization signals as determined by densitometry, and the exponent 1.3 reflects the apparent order of the hybridization reaction. C_{mRNA}, the mRNA copy number being determined, can be solved by using the following relationship:

$$C_{mRNA} = C_x (I_{mRNA}/I_x)(H_x/H_{mRNA})^{1.3}$$

where C_x, I_x, and H_x are parameters associated with a known standard, in this case the *CDC28* mRNA estimated at five to nine copies per haploid cell (30). The estimate has been revised downward from the previously published value of 6 to 12 copies per cell based on a corrected value of 925 bp for the *CDC28* mRNA coding region derived from unpublished DNA sequence data and a reanalysis of the data in reference 30. The initial underestimation of 800 bp was most likely a result of R-loop strand displacement (19). We believe that this problem has been circumvented in the present study by fixation of R-loops with glyoxal.

pressed even at low levels. R-loop analysis has the advantage of providing, in a single experiment, both the size of the gene and its precise position within the limitations of electron microscopic measurements. No prior knowledge of the gene's location or orientation is required. Intelligent application of mapping procedures using mRNA-DNA heteroduplex formation and S1 nuclease digestion (2), an alternative approach, requires at least an estimate of the gene's probable position. One additional technological adaptation we have found particularly useful is cloning into M13 vectors to determine transcriptional orientations of genes. Biological production of single-stranded DNA probes to test for mRNA complementarity is clearly preferable to the more tedious and less dependable biochemical approaches employed previously. In addition, the indirect method we describe, in which nick-translated M13 RF DNA is used as a universal labeled probe in all experiments, affords a considerable consolidation of experimental procedures.

[b] The *CDC36*-specific probe used in this experiment was the double-stranded form (RF) of M13mp8(36.3) (Fig. 3). This probe contains the entire *CDC36* coding region and therefore shares 615 nucleotides of homology with the corresponding mRNA. The standard probe used, pBR322(*CDC28*.1) (30), contains the entire *CDC28* coding region with an estimated 925 nucleotides of shared homology with the *CDC28* mRNA. The data used for the quantitation of the *CDC36* mRNA are shown in Fig. 5, in which lanes A and D correspond to experiment 3, lanes B and E correspond to experiment 2, and lanes C and F correspond to experiment 1.

[c] The *CDC37*-specific probe used in this experiment was the plasmid pKC7(*CDC37*.2). It was derived from pRC1(*CDC37*.1) by cleavage with *Eco*RI, dilution, and religation, leaving 750 nucleotides of the *CDC37* coding region. The standard probe used in this experiment was pBR322(*CDC28*.2) (30), which contains 750 nucleotides of the *CDC28* coding region.

[d] The *CDC39*-specific probe used in this experiment was the plasmid pBR322(*CDC39*.2). It was derived from pBR322(*CDC39*.1) by partial digestion with *Bam*HI and religation. A 2.5-kb segment of the *S. cerevisiae* insert segment was removed, leaving a 0.9-kb segment containing 650 nucleotides of the *CDC39* coding region. The standard probe used was pBR322(*CDC28*.2) (30), which contains 750 nucleotides of the *CDC28* coding region.

The intracellular steady-state amounts of the mRNAs corresponding to the three genes described here are remarkably similar to each other and to that of *CDC28*, a gene of analogous function, which has been described previously (30). At 1 to 10 copies per haploid cell, the probability that any of these mRNAs encodes abundant cellular proteins is low. However, without having any means to assess translational efficiencies and stabilities of the encoded products, it is impossible to estimate what polypeptide abundance the level of 1 to 10 mRNA molecules per cell might correspond to. Such information will become available only when means for direct measurement of the polypeptides in question have been developed.

As has been mentioned above, the *S. cerevisiae* genome appears to be organized extremely densely with respect to RNA coding regions (13, 16, 25, 33). This study clearly supports that view. Where adjacent coding regions were localized, the intergenic noncoding segment was either very limited or apparently nonexistent. Based on R-loop measurements, the distance between the 5′ end of the *CDC36*-coding region and the 5′ end of the coding region to the right (*TSR36* in Fig. 3) is approximately 200 bp. Presumably such spacing leaves room for the transcriptional initiation signals for both coding regions and not much else. Surprisingly, where the *CDC36*- and the *TSL36*-coding regions converge at their 3′ termini (Fig. 3), there is no detectable untranscribed region at all. R-loop measurements, in fact, indicate a small degree of overlap of the 3′ ends of the mRNAs although error analysis of the data raises some concern that this conclusion may not be highly significant. In any case, whether overlap occurs or not, we conclude that nontranscribed DNA is scarce in this region of the genome. Similarly, the noncoding segment between *CDC37* and *TSR37*, which are transcribed parallel to one another, is approximately 200 bp in length. We have not determined the length of the interval between *CDC37* and the next transcribed sequence on the left. For *CDC39*, although the exact position of the flanking transcribed sequences is not known, we have discovered that pBR322(*CDC39*.1) has complementarity to part of a second RNA species which is transcribed convergently from the right of *CDC39* (Fig. 4B). Since the position of the 3′ end of this transcript is not known, the coding region was not included in Fig. 4B. However, one can conclude that the intergenic distance is again small. Whether this extremely close spacing of genes is typical of the *S. cerevisiae* genome or peculiar to the class of genes under investigation here must await the analysis of more yeast DNA segments. Similarly tight spacing of coding regions has been observed in the segments coding for histone H2A and H2B isotypes, the *GAL1,7,10* cluster, and mating type cassettes (13, 25, 33).

We have reported that 200 bp of the *CDC37* coding region is absent from the segments described here which were capable of complementing *cdc37* mutations. We have also determined through restriction analysis that YRp7(*CDC36*.2) and YRp7(*CDC36*.3), both of which complement *cdc36* mutations, lack approximately 100 bp of the *CDC36* transcribed sequence (data not shown). These deletions are probably too large to contain only noncoding portions of the transcribed sequences. One must conclude that these polypeptides retain function even when deleted for significant carboxy-terminal segments. Similar observations have been made with the *CDC4* gene of *S. cerevisiae* (J. Yochem and B. Byers, personal communication), another gene required for cell division cycle events. It cannot be said whether such resistance to carboxy-terminal deletion is a characteristic of this special class of gene products or a more general situation.

Little can be said concerning the biochemical attributes of the products encoded by the genes described here, except that they are needed for execution of the start event and initiation of a new cell division cycle. One can infer from the sizes of the coding regions reported that the polypeptides encoded are certainly quite different in molecular weight from each other. Assuming 50 bp of nontranslated RNA per transcript and an average molecular weight of 110 per amino acid, the polypeptides encoded by *CDC36*, *CDC37*, and *CDC39* are predicted to have molecular weights of ca. 20,000, 50,000, and 100,000, respectively. The apparent molecular weight of the *CDC28* gene product has already been demonstrated to be 27,000 (30).

The detailed description of the start gene coding regions does, in fact, allow the implementation of a variety of strategies for analysis of the products of these genes and their regulation. By using RNA blotting methods similar to those described in this study, it will be possible to determine whether these genes are transcriptionally regulated. Such an approach has been successful in the analysis of the *S. cerevisiae* histone genes (15). We and others have recently shown that cloned genes can be used to prepare antisera specific for the polypeptide products these genes encode (20, 29). Our method entails fusion of a portion of the gene under investigation to a segment of the *E. coli* gene encoding β-galactosidase to produce a novel hybrid polypeptide which can be purified from the bacterial host and used as an antigen (32, 29). Antisera produced in this manner against a fusion product between β-galactosidase and the product of the

CDC28 gene were capable of immunoprecipitating *CDC28* product translated in vitro from *S. cerevisiae* mRNA. In principle, such immunological reagents should render the polypeptide product of any cloned gene, such as the ones we have described in this study, accessible to cytological and biochemical investigation. It is anticipated that important questions concerning the structural and molecular basis for control of cell division will then be resolvable.

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