Evidence That the pre-mRNA Splicing Factor Clf1p Plays a Role in DNA Replication in Saccharomyces cerevisiae

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ABSTRACT

Clf1p is an essential, highly conserved protein in S. cerevisiae that has been implicated in pre-mRNA splicing. Clf1p’s ortholog in Drosophila, Crn, is required for normal cell proliferation. Cells depleted of Clf1p arrest primarily with large buds, a single nucleus, a 2C DNA content, and a short, intact mitotic spindle. We isolated temperature-sensitive clf1 mutants that exhibit similar mitotic defects when released to the restrictive temperature from an early S-phase block. While these mutants also accumulate unspliced pre-mRNA at the restrictive temperature, the mitotic arrest does not appear to result from a failure to splice tubulin pre-mRNA. Moreover, the same mutants exhibit a delayed entry into S phase when released to the restrictive temperature from a G1 phase block. This delay could not be suppressed by disruption of the S-phase CDK inhibitor SIC1, suggesting that Clf1p is involved in DNA replication. Consistent with this possibility, we find that Clf1p (but not the mutant clf1p) interacts with the DNA replication initiation protein Orc2p in two-hybrid and co-immunoprecipitation assays, that Clf1p preferentially associates with origins of DNA replication, and that this association is Orc2p dependent. These observations suggest that Clf1p plays a direct role in the initiation of DNA replication.

TETRARTRICOPEPTIDE repeat (TPR)-containing proteins have been associated with multiple activities, ranging from control of transcription initiation to RNA processing to protein folding, modification, and proteolysis. Some TPR proteins serve as co-chaperones that may be involved in the assembly or disassembly of protein complexes responsible for such activities while other TPR proteins are thought to serve as scaffolds that help organize multisubunit protein complexes. The TPR motif contains a small number of highly invariant amino acids but otherwise can vary considerably in sequence (Lamb et al. 1995). Nevertheless, individual TPRs have a remarkably similar three-dimensional, helix-turn-helix configuration (Das et al. 1998; Gatto et al. 2000; Lapouge et al. 2000; Scheufler et al. 2000; Grizot et al. 2001; Kumar et al. 2001). Close packing of multiple adjacent TPRs results in a regular series of α-helices that forms an extended groove. In the case of the co-chaperone Hop, side chains within this groove provide the opportunity for sequence-specific and backbone contacts with the molecular chaperones Hsp70 and Hsp90 (Scheufler et al. 2000). The peroxisomal import receptor PEX5 contains two TPR clusters connected by a novel TPR hinge (Gatto et al. 2000; Kumar et al. 2001), which surrounds the peroxisomal targeting signal-1 peptide (Gatto et al. 2000). Peptide contacts with one TPR cluster are similar to the Hop-chaperone peptide contacts, while the second TPR cluster contacts the peptide in a novel manner (Gatto et al. 2000). Finally, the NADPH subunit p67phox also contains two clusters of TPRs connected by a β-hairpin insertion (Lapouge et al. 2000; Grizot et al. 2001). In this case the C terminus of the protein folds back into the TPR groove (Lapouge et al. 2000; Grizot et al. 2001), and contacts with the binding partner Rac-GTP are mediated entirely by the β-hairpin insertion (Lapouge et al. 2000).

The Drosophila Crooked neck (Crn) protein is a TPR protein that is required for normal proliferation of neuronal cell lineages (Zhang et al. 1991). Crn mutants also fail to incorporate normal amounts of BrdU, suggesting a role for Crn in DNA replication or cell cycle progression (Zhang et al. 1991). Clf1p (Crooked neck-like factor 1) is a closely related, essential TPR protein in the yeast Saccharomyces cerevisiae. Ectopic expression of Crn in yeast rescues cells that lack Clf1p, indicating that Clf1p and Crn are true orthologs (Chung et al. 1999). A growing body of evidence has implicated Clf1p in pre-mRNA splicing. For example, cells depleted of Clf1p accumulate unspliced pre-mRNAs, and addition of Clf1p to cell extracts from Clf1p-depleted cells restores pre-mRNA splicing in vitro (Chung et al. 1999; Russell et al. 2000). In addition, Clf1p was recently isolated in

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a screen for mutants that show synthetic lethality with the splicing factor Cdc40/Prp17p and are named Syf3p (Synthetic lethal with cdc forty; Ben-Yehuda et al. 2000). Clf1p/Syf3p coprecipitates with U5 and U6 snRNAs and with pre-mRNA and splicing intermediates of in vitro splicing reactions, suggesting that Clf1p associates with splicing complexes throughout the splicing reaction (Russell et al. 2000). Clf1p’s association with known and putative splicing factors also has been demonstrated by two-hybrid and in vitro co-immunoprecipitation assays (Ben-Yehuda et al. 2000; Russell et al. 2000). Two-hybrid assays have identified interactions between Clf1p/Syf3 and Syf2, Cef1p, Ntc20p, Prp22p, and Isy1p (Ben-Yehuda et al. 2000; Uetz et al. 2000) and with Mud2p and Prp40p (Chung et al. 1999). In vitro co-immunoprecipitation studies have shown that Clf1p interacts with Isy1p, Mud2p, and Prp40 (Chung et al. 1999; Ben-Yehuda et al. 2000). Finally, cd5p, the Clf1p homolog in Schizosaccharomyces pombe, was identified in a 40S snRNP-containing complex that is required for pre-mRNA splicing and contains homologs of the budding yeast proteins Cef1p, Syf1p, Prp8p, Slt11p, and Prp19p (McDonald et al. 1999). Together, these data indicate that some or all of Clf1p resides in a snRNP-containing complex essential for pre-mRNA splicing.

Several known and putative pre-mRNA splicing factors, including Clf1p/Syf3p, have also been implicated in cell-cycle progression and DNA repair. For example, both CLF1/SYF3 and SYF1 were found to be required for the G2/M transition (Russell et al. 2000; this article). In addition, Prp8p, which is required for formation of the U4/U6.U5 tri-snRNP (Brown and Beggs 1992), was independently isolated in two screens for mutants defective in the control of S phase and given the names dbf3 (dumb-bell former; Johnston and Thomas 1982a,b) and dna39 (Dumas et al. 1982). dbf3-1 mutants arrest with large buds, an ~2C DNA content, and a single undivided nucleus (Shea et al. 1994). Analysis of both dbf3 and dna39 mutants revealed an execution point after START but before the hydroxurea sensitive step of S phase (Shea et al. 1994). CEFl, like its homolog cde5+ from S. pombe, is required at the G2/M boundary (Ott et al. 1998). PRP17/CDC40 is required for proper progression through both S phase and the G2/M transition (Vaisman et al. 1995; Boger-Nadjjar et al. 1998). The function of PRP17/CDC40 during G2/M may be to help establish or maintain the mitotic spindle (Vaisman et al. 1995). cde40 mutant cells are also sensitive to methyl methanesulfonate, indicating a role for Prp17p/Cdc40p in DNA repair (Kassir et al. 1985). Finally, Ntc20p, which interacts with Clf1p in a two-hybrid assay (Uetz et al. 2000), resides in the Prp19p complex that, in addition to its probable role in spliceosome assembly, has been implicated in recombination-mediated DNA repair (da Silva et al. 1995; de Morais et al. 1996; Grey et al. 1996). It is not known whether the roles these factors play in cell-cycle control and DNA repair are carried out by splicing complexes or if instead these factors reside in multiple, functionally distinct complexes.

Clf1p also has been isolated in two-hybrid screens for proteins involved in transcriptional silencing (Cockell et al. 1998) and DNA replication (Ding 1996). These observations, together with reports implicating Clf1p in both pre-mRNA splicing and cell-cycle progression, suggested that Clf1p may be involved in multiple activities. Therefore, to further characterize Clf1p, we have isolated and characterized conditional clf1 mutants. In this article we report that one such mutant (clf1-I) exhibits pre-mRNA splicing and mitotic defects like those observed in earlier Clf1p-depletion studies (Chung et al. 1999; Russell et al. 2000). However, clf1-I mutants also exhibit a delayed entry into S phase when released to the restrictive temperature from a G1 phase block. Two-dimensional gel electrophoretic analyses indicate that clf1 mutants treated in this fashion fail to form replication bubbles with normal kinetics, suggesting a defect in replication initiation. Direct evidence of Clf1p’s involvement in replication initiation includes two-hybrid and co-immunoprecipitation assays that indicate that Clf1p interacts with the origin recognition complex (ORC). Additionally, cell fractionation studies indicate that a portion of Clf1p is chromatin associated, while chromatin immunoprecipitation assays indicate that Clf1p associates preferentially with replication origins in an Orc2p-dependent fashion. Taken together, these observations suggest that Clf1p plays a heretofore unexpected role in DNA replication.

**MATERIALS AND METHODS**

**Disruption and conditional expression of CLF1:** A full-length genomic copy of CLF1 was isolated from a partial SacAI genomic library (2J351; provided by J. Hirsch and J. Kurjan; Engelbrecht et al. 1990) by screening with 32P-labeled CLF1 cDNA and washing filters at a stringency of 10–15° below the predicted Tm, as described (Pederson et al. 1984). The 6.6-kbp genomic clone, YEP351-CLF1, includes 1290 bp 5′ of the initiator methionine, the CLF1 open reading frame itself, and 3039 bp 3′ of the CLF1 termination codon. To disrupt CLF1, we subcloned the 6.6-kbp fragment containing CLF1 from YEP351-CLF1 into pRS314 (Sikorski and Hieter 1989), creating pRS314-CLF1, and replaced a 500-bp BglII fragment within CLF1 with a BglII fragment containing LEU2 DNA. A Smal-PvuII fragment encompassing the clf1::LEU2 fusion was isolated from the resulting plasmid and transfected into the diploid strain W303. Leu+ transformants were analyzed by Southern blotting to ensure the presence of the disruption alleles, and one such transformant was designated as strain WCY1 (Table 1). To construct a conditionally expressed allele of CLF1, we first inserted into the EcoRI-BamHI site of the URA3-CEN4-containing vector pGAL (Kang et al. 1990) a 74-bp EcoRI-BamHI fragment encoding an initiator methionine, the 12-amino-acid hemagglutinin (HA) epitope, and a 12-amino-acid linker, creating the plasmid pGAL-HA. We then inserted into pGAL-HA a PCR-derived BamHI fragment containing the CLF1 open reading frame immediately 3′ of the HA DNA, forming the plasmid pGAL-HA-CLF1. CLF1::clf1::LEU2

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diploids (strain WC1) were transformed with pGal-HA-CLF1. The resulting diploids were sporulated, and Leu+ Ura+ hapl- loid progeny were isolated on galactose-containing media. Haploidy was confirmed using an α-factor halo assay (Sprague 1991) with the indicator strains TCK1 and TN44-1B.

Immunofluorescence and FACS analyses: Tubulin staining was performed essentially as described (Adams and Pringle 1984) using the rat monoclonal antitubulin antibody YO1/34 (Accurate Chemical and Scientific, Westbury, NY; Kilmartin et al. 1982; Kilmartin and Adams 1984) and an FITC- anti-rat secondary antibody (Jackson Immunoresearch, West Grove, PA; P/N 112-095-062). Cells were mounted in 4% diamidino-2-phenylindole (DAPI)-containing media (2.25 μg/ml) and examined using a Nikon E400 microscope with phase contrast optics (Omega Optical, Brattleboro, VT). Video images were obtained using a VE1000ST camera (Dage-MTI, Michigan City, IN) and digitized using an LG-3 video frame grabber card (Scion, Frederick, MD). FACS analyses were carried out as described (Haase 1997).

Isolation of temperature-sensitive clf1 alleles: CLF1 was sub- jected to error-prone PCR mutagenesis, essentially as de- scribed (Muhlrad et al. 1992). Reactions of 0.1 ml included 10 ng pRS314-CLF1, 6 mM MgCl2, 0.65 mM MnCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.001% gelatin, 0.2 mM dATP, 1 mM each dGTP, dCTP, and dTTP, 0.2 μM of a primer annealing 100 bp 5′ of the CLF1 initiation codon, and 0.2 μM of a primer annealing 1000 bp 3′ of the CLF1 terminus codon. Reactions were initiated by addition of 0.5 units of Taq Poly- merase (Perkin-Elmer, Norwalk, CT) in a hot start procedure and continued for 30 cycles. A gapped plasmid was prepared by removing from pRS314-CLF1 a Bsr-BglII DNA fragment beginning 68 bp 3′ of the CLF1 initiation codon and ending 588 bp 3′ of the CLF1 stop codon. Approximately 400 ng of the gapped plasmid and 20 μl of the PCR product were cotransformed into strain MD103. Transformants were selected at 23°C, and 2544 of these were transferred to 96-well microtiter plates. Cell suspensions were spotted onto plates containing 1 mg/ml 5-fluoroorotic acid (5-FOA; USB) to select for loss of pGal-HA-CLF1. After 3–4 days of growth, 5-FOA- resistant colonies were replica plated onto selective plates and scored for growth at 15°C, 23°C, 30°C, and 37°C. Plasmids were recovered from 10 of the 13 transformants that showed little or no growth at 37°C and retransformed into strain MD103. A second plasmid shuffle was carried out with these trans- formants to confirm the ability of selected plasmids to confer temperature sensitivity on the resulting clf1::LEU2 cells. One strain containing the plasmid-borne temperature-sensitive al- lele was determined to be recessive by transformation with wild-type CLF1 and was designated IRP22A3a (Table 1). The same clf1-1 allele also was integrated into Chromosome XII by subcloning the 6.6-kb Nol-KpnI fragment containing clf1-1 into pRS306 (Sikorski and Hieter 1989). The resultant clone, pRS306-clf1-1, was linearized with NruI at a site 1027 bp down- stream of the CLF1 stop codon and transformed into the diploid CLF1/clf1::LEU2 strain WC1. Transformants were sporulated and tetrads were dissected. Leu+, Ura+ spores that failed to grow at 37°C were analyzed by Southern blotting to confirm integration of a single copy of pRS306-clf1-1, and one such segregant was designated strain IR22A3a (Table 1).

Two-hybrid studies: To construct an Orc2p bait protein, we PCR amplified a DNA fragment containing the ORC2 open reading frame using genomic DNA from strain T4D and oligo- nucleotide primers 5′-CCGCCTGAGTGATATACAGAGATTTA AAACGGTTTT-3′ and 5′-CCGGAGTCGATCCATGAGAACGG GAAAGCTTT-3′ (Micklem et al. 1993). The resulting frag- ment was cleaved with XhoI and BamHI, gel purified, and inserted into the TRPI-containing vector pAS-CYH1, cleaved with SalI and BamHI (supplied by S. J. Elledge). DNA sequenc- ing demonstrated that the resulting plasmid, pAS1-ORC2, en- codes a fusion protein consisting of the Gal4p DNA-binding domain at the N terminus, linked in frame to the HA peptide epitope and to Orc2p. Evidence that this fusion protein func- tions as Orc2p in vivo is described in results. CLF1 and clf1-1 coding regions were PCR amplified using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) and the primers 5′-CGG GATCCGAGATGAGAGCATTTATAGGCAAC-3′ and 5′-CCGGATC TCAAAAGTGCCTTTCTCTCTTTCTC3′. The resulting fragments were digested with BamHI and cloned into BamHI-digested pACTII, to generate in-frame fusions of the HA epitope with CLF1 or clf1-1. Transformants were plated onto complete syn- thectic dextrose (SD) media lacking his, trp, and leu (Sherman et al. 1983), transferred to fresh SD media lacking his, trp, and leu, and tested for lacZ activity using a colony filter assay.

Co-immunoprecipitation and chromatin-fractionation studies: To prepare immune precipitates, ~106 cells were col- lected, washed with water, and resuspended in approximately one packed cell volume (0.7 ml) of breakage buffer: 25 mM HEPES-KOH pH 7.6, 100 mM KCl, 1 mM MgCl2, 50 mM NaCl, 0.05 mM dithiothreitol (DTT), 0.1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM [N-tosyl- l-phenylalanine chloromethyl ketone (TPCK)], 1 μg/ml leu- peptin, and 1 μg/ml pepstatin A. The cell slurry was mixed with 1/2 volume of acid-washed glass beads, and cells were lysed by eight cycles of vortexing for 30 sec followed by incuba- tion on ice for 30 sec. Beads and cell debris were removed by centrifugation, and the resulting supernatants were mixed with 25–50 μl anti-HA.11-crosslinked Protein A-Sepharose beads (BabCo, P/N AFC-101P). After allowing immune complexes to form for 2–3 hr at 4°C, beads were collected by low-speed centrifugation for 5–10 sec and washed six times with 1.0 ml of extraction buffer. Associated proteins were eluted by incubating beads in Laemmli buffer at 85°C for 10 min, separated on a 7–18% SDS/PAGE gradient gel, transferred to Immobilon membranes (Millipore, Bedford, MA), and visual- ized using antibodies to yeast Orc2p (kindly provided by Bruce Stillman). The chromatin-fractionation assay was performed as described (liang and Stillman 1997) with minor modifications. Approximately 106 mid-log phase cells were treated with 0.1% NaCl, harvested, and resuspended in 0.25 M sorbitol in vivo in Laemmli buffer for 2 min at 23°C. Nuclei were washed in extraction buffer and centrifuged at 23°C, suspended in 0.25 volumes extraction buffer for 2 min at 23°C, and recovered from 10 of the 13 transformants that showed little prespheroplasting buffer (50 mM KH2PO4, pH 7.5, 10 mM DTT) for 10 min at 23°C. Cells then were treated with 0.8 mg/ml zymolase (100T, Sigma, St. Louis) in prespheroplasting buffer supplemented with 0.8 M sorbitol. The resulting spheroplasts were washed in 1 ml ice-cold wash buffer (100 mM KCl, 50 mM HEPES-KOH pH 7.5, 2.5 mM MgCl2, 0.6 M sorbitol) and lysed in ice-cold extraction buffer (100 mM KCl, 50 mM HEPES- KOH (pH 7.5), 2.5 mM MgCl2, 50 mM NaF, 5 mM Na3P04, 0.1 mM NaVO3, and 0.25% Triton X-100), containing freshly added 0.5 mM PMSF, 0.05 mM TPCK, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A. The resulting whole-cell extracts (fraction 1, Figure 6) were layered onto 0.5 volumes of 30% sucrose and centrifuged at 11,600 × g for 10 min at 4°C to pel- let nuclei and recover cytoplasmic contents in the supernatant. Nuclei were washed in extraction buffer and centrifuged at 8000 × g for 5 min at 4°C, suspended in 0.25 volumes extraction buffer for 2 min at 23°C, and treated with 600 units of micrococcal nuclease (MNase; Worthington Biochemical, Lakewood, NJ) in the presence of 1 mM CaCl2 for 4 min. Digestion was stopped by addition of EGTA to 1 mM, and the mixture was centrifuged at 8000 × g for 2 min at 4°C. Nuclei were suspended and treated a second time with 200 units MNase, and the postnuclease supernatants were combined. A portion of the combined supernatants was centrifuged at 100,000 × g for 1 hr at 4°C to pellet chromatin released by MNase treatment, together with any associated proteins. Equiva-
lent portions of each fraction were loaded onto 7.5% SDS-PAGE gels and Western blots were probed with the anti-HA antibody 12CA5.

**Chromatin immunoprecipitation assay**: Chromatin immunoprecipitation (ChIP) assays were conducted as described (Strahl-Bolsinger et al. 1997) with minor modifications (Hecht and Grunstein 1999; Meluh and Broach 1999). Briefly, ~10^6 mid-log phase cells were treated with 1% formaldehyde for 10 min at room temperature (~25°C). The crosslinking reaction was quenched by addition of glycine to 0.125 M. After 6 min incubation at 23°C, cells were collected, washed twice in cold (4°C) PBS, and suspended in 0.4 ml cold lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% NaDeoxycholate), containing freshly added protease inhibitors (25 μM PMSF, 25 μM TPCK, 1 μg/ml each peptatin and leupeptin). Cells were lysed by vortexing (eight times for 30 sec each) in the presence of 0.4 ml acid-washed glass beads. The cell lysate was subject to microcentrifugation for 5 min, and the insoluble chromatin-containing pellet was suspended in 0.4 ml lysis buffer and sonicated (four times for 30 sec each) using a Virsonic 100 sonicator and microtip, at a power setting of 3 (out of 5). The sonicate was cleared by microcentrifugation for 5 min at 4°C and mixed with the anti-HA.11 antibody coupled to Sepharose beads (prewashed three times with lysis buffer). After 2–16 hr at 4°C, immunoprecipitates were collected by low-speed centrifugation and washed at 23°C with 1.3 ml each of lysis buffer, lysis buffer containing 0.5 M NaCl, ChiP wash buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 0.5% NP40, 0.5% NaDeoxycholate, and 1 mM EDTA), and finally twice with TE (pH 8.0). The beads next were suspended in 0.2 ml TE and incubated first with 40 μg/ml RNase A for 30 min at 37°C, and then with 0.25% SDS and 250 μg/ml proteinase K for 60 min at 50°C. Crosslinked DNA was released by further incubation for 6 hr at 65°C, purified by extraction with phenol and CHCl₃, and ethanol precipitated.

DNA was suspended in TE buffer and used in PCR reactions described in the legend to Figure 9.

**RESULTS**

**Arrest phenotype of cells lacking Clf1p**: Mutation of Ctn, the Clf1p homolog in *Drosophila melanogaster*, results in poor incorporation of BrdU and defective proliferation of neuroblast lineages, suggesting a role for Ctn in DNA replication or cell-cycle progression (Zhang et al. 1991). To investigate Clf1p’s cell-cycle role in yeast, we first examined the phenotype of cells depleted of Clf1p. MD103 cells, in which the sole copy of *CLF1* is expressed from the *GAL1,10* promoter, were grown to early log phase in galactose or a mixture of raffinose and galactose. Cells then were washed and suspended in glucose-containing media and examined by microscopy and FACS at regular intervals. Cell numbers continued to increase for ~10 hr (four to six cell generations) after the shift from galactose- to glucose-containing media. At this point, ~60% of the cells had large buds that continued to elongate with prolonged incubation. FACS analyses (Figure 1A) indicated that the fraction of cells containing 2C DNA increased progressively with the progressive depletion of Clf1p. DAPI staining after 12 hr of depletion indicated that cells contained a single nucleus, most often located in the mother cell (Figure 1B). In some cells, however, the nucleus was elongated and located partially in the neck between the mother...
and daughter bud. Tubulin staining indicated that most of the visible spindles in the large-budded, Clf1p-depleted MD103 cells were less than one-half the length of anaphase spindles (Figure 1B). Thus, cells depleted of Clf1p arrest predominantly in metaphase with intact, fully formed spindles.

In some instances, defects in pre-mRNA splicing result in mitotic arrest. For example, cells defective in Prp22p, an RNA helicase involved in splicing, arrest in mitosis without spindles (Hwang and Murray 1997), most likely due to the failure to splice TUB1 and TUB3 pre-mRNAs. The subsequent failure of these cells to assemble a mitotic spindle would activate the spindle checkpoint, which arrests cells in mitosis. By contrast, cells depleted of Clf1p arrest with intact, fully formed spindles. Thus, while cells depleted of Clf1p also accumulate unspliced pre-mRNAs (Chung et al. 1999; Ben-Yehuda et al. 2000; Russell et al. 2000; Figure 3B, this article), it is unlikely that defects in tubulin pre-mRNA splicing account for their arrest in mitosis. To test this inference further, we disrupted the MAD2 (mitotic arrest deficient) gene in a clf1 mutant that, as described below, fails to progress through mitosis at the restrictive temperature. Cells defective in MAD2 are unable to activate the spindle checkpoint (Li and Murray 1991). If the arrest of cells lacking functional Clf1p was due to a spindle defect, we predicted that inactivating the spindle checkpoint would lead to aberrant chromosome segregation and decreased survival rates. However, as shown in Figure 2, the viability of clf1-1 mad2A cells held at the restrictive temperature for up to 6 hr was comparable to that of the clf1-1 single mutant. We also reasoned that if Clf1p were involved in establishing or maintaining spindle integrity, clf1 mutants might be aberrantly sensitive to microtubule destabilizing agents. However, clf1 mutants did not display elevated lethality when treated with taxol at the permissive temperature (data not shown). Likewise, both wild-type cells and clf1 mutants were able to form colonies at the permissive temperature on plates containing 5 μg/ml benomyl whereas growth of cells containing a disrupted MAD2 gene was impaired (data not shown). Interestingly, clf1 mad2 double mutants were less sensitive to 5 μg/ml benomyl than were the mad2 single mutants. The mitotic delay associated with the clf1 mutant may provide mad2 cells the added time that would normally be provided by activation of the MAD2 checkpoint. Collectively, these observations suggested that spindle defects do not account for the mitotic arrest of Clf1p-depleted cells. This in turn suggested that the role of Clf1p in cell-cycle progression is not restricted to pre-mRNA splicing.

**Isolation and characterization of temperature-sensitive clf1 mutants:** To investigate the possible involvement of Clf1p in activities unrelated to pre-mRNA splicing, we isolated a temperature-sensitive allele of CLF1, desig-
Survival of $clf1-1$ mutants at the restrictive temperature does not depend on an intact spindle checkpoint pathway. $clf1-1$ mad2Δ double mutants and the corresponding single mutants were grown to mid-log phase at 23°C and then shifted to the restrictive temperature (37°C). Cell aliquots were removed at regular intervals, and equal numbers were plated onto rich media and allowed to form colonies at 23°C. Survival curves shown represent the average of multiple independent experiments in which the number of viable cells was divided by the number of viable cells at time zero. ((circle) $clf1-1$; (square) mad2Δ; (triangle) $clf1-1$ mad2Δ.

As shown in Figure 3A, $clf1-1$ mutants are unable to grow at 37°C. The temperature-sensitive growth phenotype could be suppressed by the addition of CLF1 on a CEN-containing plasmid, but not by the addition of either $clf1-1$ on a CEN plasmid or the parental vector alone, indicating that $clf1-1$ is recessive. To determine if $clf1-1$ mutants are defective in pre-mRNA splicing, we isolated RNA from $clf1-1$ mutants before and after shifting cells to the restrictive temperature. As a control, we also isolated RNA from cells depleted of Clf1p, as was done in studies that first implicated Clf1p in pre-mRNA splicing (Chung et al. 1999). As expected, Northern blot analyses in Figure 3B show that a slower-migrating, unspliced form of RP51A mRNA accumulated in $clf1p$-depleted cells. The same slower-migrating, unspliced pre-mRNA also was evident in $clf1-1$ cells held at the restrictive temperature, but not at the permissive temperature, nor was it evident in wild-type cells at either temperature (Figure 3B). Thus, the pre-mRNA processing defect evident in the Clf1p-depletion studies also occurs in $clf1-1$ mutants.

Clf1p functions at multiple points in the cell cycle: If the cellular concentration at which a multifunctional protein becomes rate limiting is higher for one activity than for a second activity, a depletion study may reveal only one of two or more execution points in the cell cycle. This proved to be the case for CLF1. Microscopic examination indicated that $clf1-1$ cells arrest within a single cell generation after being shifted to the restrictive temperature but fail to show a clear cdc phenotype. To determine if the mixture of terminal phenotypes was due to a requirement for Clf1p at multiple discrete points in the cell cycle, we first blocked $clf1-1$ cells in early S phase with hydroxyurea (HU) and then simultaneously removed the HU and shifted cells to the restrictive temperature. FACS analyses in Figure 4A show that DNA replication was complete within 30 min of release from the HU block; however, cells remained arrested with a 2C DNA content for the duration of the experiment (120 min). At this point, 62% of $clf1-1$ cells had large buds, 22% had small buds, and the remaining...
16% were unbudded. These results were consistent with the Clf1p-depletion studies, which indicated that CLF1 is required for progression through mitosis.

To determine if Clf1p is required for cell-cycle progression outside of mitosis, we next blocked wild-type and clf1-1 cells in G1 phase with α-factor and released them to the restrictive temperature. FACS analyses of wild-type cells (Figure 4A) showed a gradual increase in DNA content beginning 20–40 min following release from the α-factor block, with the development of a clear 2C peak 60–80 min after release; an increase in the 1C peak 100–120 min after release signaled the onset of cell division. By contrast, the 1C DNA peak in clf1-1 mutants persisted far longer and, while the average DNA content gradually increased over the duration of the experiment, cells failed to develop a clean 2C peak within the 2-hr time frame of the experiment and showed no evidence of cell division. Microscopic examination showed an ∼60 min delay in bud formation in clf1-1 mutants released to the restrictive temperature.
relative to the kinetics of bud formation at the permissive temperature (Figure 4B). Most clf1-1 mutants formed large buds within 3 hr after release but then failed to divide. The ~1-hr delay in the onset of bud formation evident at 37°C also was evident at 38°C, 39°C, and 40°C (not shown), suggesting that the eventual onset of bud formation and entry into S phase is not the result of a leaky allele. Thus, clf1-1 mutants fail to enter S phase with normal kinetics at the restrictive temperature, suggesting that Clf1p plays a role not only during mitosis but also during late G1 phase or at the G1/S transition.

The reciprocal shift experiments described above suggested that Clf1p plays a role either in replication initiation or in some late G1 phase cell-cycle event unrelated to DNA replication. For example, synthesis of the G1 phase cyclins following the release of cells from an α-factor block leads to the phosphorylation and subsequent proteolysis of the S-phase CDK inhibitor Sic1p (reviewed by Tyers and Willems 1999). Disruption of SIC1 enables cells that lack G1 phase cyclins to enter S phase (Schneider et al. 1996; Tyers 1996). Therefore, to determine if Clf1p is involved in the synthesis or activation of the G1 phase CDKs, or in the destruction of Sic1p, we disrupted SIC1 in clf1-1 mutants and in the wild-type parental strain. Because cells lacking Sic1p could not be easily synchronized with α-factor, we examined the effect of SIC1 disruption in nonsynchronously growing cells (Figure 5). The majority of sic1Δ single mutants were large budded, reflecting an abnormally slow progression through mitosis, and were unaffected by a shift from 23° to 37°. Cells are slow to exit mitosis because Sic1p ordinarily helps to inhibit Clb-CDK activity and thereby promote mitotic exit; in the absence of Sic1p, cells must rely on anaphase promoting complex-mediated degradation of the Clb cyclins to exit mitosis (Donovan et al. 1994; Schwab et al. 1997; Visintin et al. 1997). At the permissive temperature, the fraction of large-budded clf1-1 sic1Δ double mutants was similar to that of the sic1Δ single mutants. However, when the clf1-1 sic1Δ double mutants were shifted to the restrictive temperature, the fraction of budded cells declined. The corresponding increase in un budded cells indicated that the clf1-1-associated delay in entry to S phase persists even in the absence of Sic1p. These observations indicated that Clf1p’s G1 phase role does not involve (or is not restricted to) activation of CLN kinases or proteolysis of Sic1p.

clf1-1 mutants fail to initiate DNA replication with normal kinetics: The failure of clf1-1 mutants to bud and accumulate DNA with normal kinetics at the restrictive temperature, and the fact that disruption of SIC1 failed to suppress this defect, led us to ask whether Clf1p is required for the initiation of DNA replication from

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**Figure 5.** Disruption of SIC1 fails to suppress the delayed entry into S phase evident in clf1-1 mutants. sic1Δ ( ), clf1-1 ( ■), and sic1Δ clf1-1 ( ▲) mutants were grown to mid-log phase at 23°C and examined by microscopy before and at regular intervals after shifting to the restrictive temperature (37°C). Within 2 hr after shifting cultures to 37°C, the fraction of unbudded sic1Δ clf1-1 cells increased to that observed for clf1-1 single mutants, indicating that disruption of SIC1 failed to suppress the clf1-1-associated delay in entry into S phase.

**Figure 6.** clf1-1 cells fail to form replication intermediates with normal kinetics at the restrictive temperature. W303-1A; bar1::HIS3 (wild type) and clf1-1 bar1::HIS3 (strain WZY002; clf1) cells were blocked in G1 phase with α-factor and then released to either the permissive temperature of 23°C or the restrictive temperature of 37°C. Cell aliquots were collected at 30, 40, 50, and 60 min after release and pooled. High molecular weight DNA was prepared from the pooled cells and digested with NotI. Replication intermediates were enriched using benzoylated napthoylated DEAE cellulose (Liang et al. 1993) and analyzed by two-dimensional gel electrophoresis as described (Brewer and Fangman 1987; http://fangman-brewer.genetics.washington.edu/2Dgel.html). ARS1-associated replication intermediates were visualized by Southern blotting. The position of arcs formed by replication bubbles are indicated by arrows.
chromosomal replicators. To address this question, clf1-1 mutants and wild-type parental cells were arrested in mid-log phase in G1 phase with α-factor and released into fresh media at permissive or nonpermissive temperature. Cell aliquots were collected at 30, 40, 50, and 60 min after release from the G1 block, an interval that encompasses the normal time of firing of ARS1 (Brewer and Fangman 1987; H. Pan and D. S. Pederson, unpublished observations). Samples were combined and high molecular weight DNA was prepared and cleaved with NcoI. Restriction fragments containing ARS1 replication intermediates were enriched by BND cellulose chromatography and examined by neutral–neutral two-dimensional gel electrophoresis and Southern blotting (Brewer and Fangman 1987). Figure 6 shows that replication bubbles associated with ARS1 form in the wild-type strain at both 23° and 37° and in the clf1-1 strain at 23°. By contrast, replication bubbles failed to form within the normal time frame in clf1-1 mutants held at the restrictive temperature. Thus, CLF1 is required for efficient replication initiation.

Physical interactions between Clf1p and the DNA replication complex ORC: The evidence outlined above suggests that Clf1p’s G1 phase role is related to the initiation of DNA replication rather than cell-cycle progression, an inference consistent with our having isolated Clf1p in a two-hybrid screen for proteins involved in DNA replication (Ding 1996; Figure 7B, this article). The ORC helps recruit factors necessary for DNA replication, and ORC mutants show defects in progression
subunits or ORC-associated factors. Compare MNase soluble and insoluble fractions in direct interactions between Clf1p and Orc2p as well MNase or DNase I (see below); mock digestion failed in vivo is unable to grow at temperatures restrictive for growth not treated with MNase in lanes 4 in that the bait protein is at least partially functional orc2-1 and the empty vector into an active ORC in vivo act with an Orc2-Gal4p DNA-binding domain fusion that Clf1p interacts physically with Orc2p and that ORC-dependent association of Clf1p with chromatin possibility, we generated Clf1- and clf1-1-Gal4p activate that Clf1p interacts physically with Orc2p and that kinetics at the restrictive temperature might be due to function at the permissive temperature. In sum, both of clf1-1 mutants, we assayed Clf1p-Orc2p interactions at the permissive temperatures of 23° and 30° and at the semipermissive temperature of 32°. The two-hybrid results were identical at all temperatures, and results for 23° and 30° are shown in Figure 6B. β-Galactosidase activity was detectable when the Orc2p-binding domain fusion was paired with the Clf1p-activation domain fusion but not when it was paired with the clf1p-activation domain fusion, even at the permissive temperature of 23°. These results suggested that Orc2p-Clf1p interactions are important for cell-cycle progression.

To further investigate the significance of the two-hybrid interactions between Clf1p and ORC, we tested whether Clf1p would co-immunoprecipitate with Orc2p. Extracts were prepared from wild-type cells and from cells in which a chromosomal copy of CLF1 or clf1-1 was N-terminally tagged with three copies of the HA epitope and expressed from its native promoter. Clf1p and clf1-1p-associated proteins were co-immunoprecipitated using anti-HA-crosslinked Sepharose beads and immunoblotted with antibodies to Orc2p. Significant amounts of Orc2p were evident in immunoprecipitates prepared from cells expressing HA-Clf1p (Figure 7C, compare lanes 4 and 6). A smaller fraction of Orc2p was evident in immunoprecipitates prepared from cells expressing HA-clf1-1p (Figure 7C, compare lanes 7 and 9). Thus, interactions between clf1-1p and ORC are weaker than those between Clf1p and ORC, in accord with the absence of a detectable two-hybrid interaction between clf1-1p and ORC. Western blotting of the wild-type and mutant proteins indicated that this result is not due to a lower cellular concentration of clf1p (Figure 7D). Presumably, the residual, weak interactions between clf1-1p and ORC are sufficient to permit clf1-1 mutants to function at the permissive temperature. In sum, both the two-hybrid and co-immunoprecipitation assays indicate that Clf1p interacts physically with Orc2p and that this interaction is functionally significant.

**ORC-dependent association of Clf1p with chromatin and replication origins:** ORC binds ARS DNA in vitro and in vivo (Bell and Stillman 1992; Bell et al. 1993; Difffley et al. 1994; Liang and Stillman 1997; Tanaka et al. 1997; Geraghty et al. 2000). To test whether Clf1p is also chromatin associated, we prepared nuclei from wild-type cells containing an HA-tagged chromosomal copy of ORC1 and an episomal copy of HA-CLF1. Although Clf1p was present in both the cytoplasmic and nuclear fractions (Figure 8, lanes 2 and 3, respectively), Orc1p was present in both fractions as well. Thus, some or all of the cytoplasmic Clf1p may result from leakage during preparation of nuclei. Elution of additional Clf1p and Orc1p from nuclei required treatment with MNase or DNase I (see below); mock digestion failed to release appreciable amounts of either protein (Figure 8; compare MNase soluble and insoluble fractions in lanes 4 and 5 to the equivalent fractions from nuclei not treated with MNase in lanes 4’ and 5’). As with
Orc1p, much of the Clf1p released from MNase-treated nuclei could be pelleted by high-speed centrifugation (Figure 8, lane 7), as would be expected for a chromatin-associated protein. By contrast, an endogenous, cross-reacting protein (indicated in Figure 8 by an open arrowhead) did not sediment during high-speed centrifugation. These results suggested that at least a portion of Clf1p is chromatin-associated. However, the ability of MNase to digest RNA as well as DNA, together with the likely involvement of Clf1p in pre-mRNA splicing, raised the possibility that the Clf1p released upon treatment of nuclei with MNase was due to release of Clf1p from ribonucleoprotein complexes rather than from chromatin. To rule out this possibility, we treated nuclei with DNase I, which does not cleave RNA, and found that Clf1p could also be released from nuclei by digestion with DNase I (data not shown).

To test whether Clf1p is associated with origins of DNA replication, we used a ChIP assay (Hecht et al. 1996; Strahl-Bolsinger et al. 1997; Meluh and Broach 1999). Cells from strain IRH206 containing HA-tagged CLF1 and from strain OAy503 containing HA-tagged ORC1 were treated with formaldehyde to crosslink proteins to one another and to DNA. Chromatin was sheared by sonication, and DNA associated with either CLF1 or ORC1 was separately co-immunoprecipitated using an anti-HA antibody. After reversing the protein-DNA crosslinks, immunoprecipitated DNA was purified and used in PCR amplification reactions along with primers specific for the H4-associated ARS (“H4-ARS”). As a control, we used primers that anneal to a segment of the SIC1 gene, which does not contain an ARS. Figure 9A shows that ARS DNA is preferentially immunoprecipitated in cells expressing HA-ORC1p, as previously reported (Aparicio et al. 1997). Importantly, ARS DNA is also preferentially immunoprecipitated in cells expressing HA-Clf1p. Thus, Clf1p is associated with origins of DNA replication. Additional ChIP assays shown in Figure 9A indicate that Clf1p associates with both early and late-firing origins of DNA replication (ARS305 and ARS501, respectively).

While the ChIP studies in Figure 9A indicate that Clf1p is ARS associated, they do not indicate whether Clf1p binds DNA directly or through its association with known replication factors. Given that Clf1p physically interacts with ORC, we asked whether Clf1p’s association with ARS is ORC dependent by introducing the HA-CLF1 allele into orc2-1 temperature-sensitive mutants. At the restrictive temperature, orc2-1p-containing ORC complexes no longer bind to ARS DNA in vitro (Bell et al. 1993). Figure 9B shows that Clf1p is lost from ARS305 DNA after incubating orc2-1 cells for 90 min at the restrictive temperature. This result suggested that ORC either recruits or helps stabilize the binding of Clf1p to ARS DNA. These findings provide strong evidence that the physical association of Clf1p with replication origins is functionally important. This, along with the replication defects evident in clf1 mutants, strongly suggests that Clf1p plays a direct role in the initiation of DNA replication.

**DISCUSSION**

Clf1p associates with protein and snRNA components of splicing complexes and appears to play a direct role either in pre-mRNA splicing or in the assembly of splic-
ing complexes (Chung et al. 1999; Ben-Yehuda et al. 2000; Russell et al. 2000; Uetz et al. 2000). As with several other members of the splicing complex, Clf1p is also required for cell-cycle progression (Vaisman et al. 1995; Boger-Nadjar et al. 1998; Russell et al. 2000).

Our work confirms earlier observations that cells depleted of Clf1p not only accumulate unspliced pre-mRNA but also are unable to complete mitosis, arresting with large buds, duplicated DNA, and a single nucleus (Russell et al. 2000; this article). A potential concern in the Clf1p-depletion studies is that cells continue to grow for several generations after the shift from galactose- to glucose-containing media before arresting in mitosis due to the loss of Clf1p. This makes it difficult to determine if defects evident in Clf1p-depleted cells are the primary cause of cell-cycle arrest. Indeed, although Clf1p-depleted cells accumulate unspliced mRNA, several observations suggest that Clf1p-depleted cells arrest in mitosis for some reason other than their failure to splice tubulin pre-mRNAs. Specifically, antitubulin staining of Clf1p-depleted cells revealed short, intact spindles indicative of a metaphase arrest. Because the tubulin genes TUB1 and TUB3 each contain an intron, the presence of a mitotic spindle was inconsistent with a general pre-mRNA splicing defect. Moreover, clf1-1 mutants are not exceptionally sensitive to microtubule destabilizing agents, and the observed metaphase arrest did not appear to depend on an intact spindle checkpoint pathway. These observations suggested that the failure of Clf1p-depleted cells to complete mitosis is not due to a general defect in pre-mRNA splicing and prompted us to further investigate the role of Clf1p in cell-cycle progression.

Our reciprocal shift experiments with clf1-1 confirmed the requirement for CLF1 during mitosis and revealed an additional requirement for CLF1 at or near the G1/S transition. Specifically, clf1 cells blocked in G1 with α-factor and released to the restrictive temperature fail to enter S phase with normal kinetics; after a pronounced lag, most cells initiate bud formation and eventually arrest with large buds. The delayed entry into S phase is reminiscent of that observed following mutation of a number of other proteins required for the initiation of DNA replication, notably Cdc6p, Cdc45p, and Sld3p (Liang and Stillman 1997; Zou et al. 1997; Kamimura et al. 2001). clf1 mutants released from an α-factor block to the restrictive temperature showed a gradual shift to higher average DNA content but failed to develop a clean 2C DNA peak within the 2-hr period examined. This phenotype, and the accompanying progressive decline in cell viability, is reminiscent of that observed for orc2-1 (Bell et al. 1993). We also determined that Clf1p’s role in G1 does not involve (or is not restricted to) degradation of the S-phase inhibitor Sic1p. These observations, together with our having previously isolated Clf1p in a two-hybrid screen for ORC-interacting proteins, were consistent with a role for Clf1p in replication initiation. We have presented several additional lines of evidence that support this inference. First, although wild-type Clf1p interacts with Orc2p in a two-hybrid assay, clf1-1p does not. Additionally, the interaction between clf1-1p and Orc2p in an immunoprecipitation assay is less robust than it is for wild-type Clf1p. Second, a portion of Clf1p cofractionates with Orc1p and preferentially associates with ARS DNA. Finally, the association of Clf1p with ARS DNA is ORC dependent. Collectively, these results indicate that Clf1p-ORC interactions are functionally important and suggest that Clf1p plays a role in the initiation of DNA replication.

The likely involvement of Clf1p in DNA replication probably accounts for the delayed onset of S phase in clf1-1 mutants at the restrictive temperature. It is not yet clear if the mitotic arrest that occurs in clf1-1 mutants is also related to DNA replication but it is noteworthy that, like clf1-1, conditional orc5 mutants also arrest at both the G1/S transition and in mitosis. The ORC execution point occurs as cells exit mitosis, which coincides with the point when prereplicative complexes (preRCs) begin to assemble at origins of DNA replication. The failure to form preRCs presumably leads to a G1/S arrest in orc5 mutants released from an M-phase block. The fact that both CLF1 and ORC5 are required at G1/S and M phase, together with our evidence of physical interactions between Clf1p and ORC, suggest that Clf1p may play a role in preRC formation. We are currently testing this possibility.

Mutation of most known replication factors increases the rate of plasmid loss from cells grown in nonselective media. Given the evidence of Clf1p’s involvement in DNA replication, we were surprised to find that plasmid loss rates in clf1 mutants grown at permissive and semi-permissive temperatures (23° and 30°, respectively) were similar to those in wild-type cells, as measured by conventional means and by fluctuation tests (data not shown). Because it was possible that increased plasmid loss would occur only at the restrictive temperature, we also monitored plasmid loss in clf1 mutants exposed to 37° for 3 hr, using a colony-sectoring assay similar to that described by Hogan and Koshland (1992). Once again, plasmid loss rates were similar to those in wild-type cells. However, two observations raised a concern about the validity of these measurements. First, many of the clf1 cells in this experiment might be protected from plasmid loss due either to their arrest in mitosis or to delayed entry into S phase. Additionally, as shown in Figure 2, the plating efficiency of clf1 cells incubated for 3 hr at the restrictive temperature was less than half that of clf1 cells grown at 23° or of wild-type control cells. If the cells that die do so because of aberrant DNA replication, then cells most likely to suffer plasmid loss would be underrepresented among the colonies that form after plating. If this explanation for our plasmid loss results
were correct, we might find that clf1 mutants activate DNA damage or replication checkpoints at the restrictive temperature. To test this prediction, we introduced the clf1-1 mutant into cells mutated for rad53, rad9, or mec1. None of the resulting double mutants exhibited synthetic lethality. Additionally, the capacity of the double mutants to survive incubation at the restrictive temperature for up to 6 hr was similar to or only moderately (two- to threefold) less than that of the corresponding single mutants. These results suggested that clf1 mutants do not accumulate significant amounts of DNA damage at the restrictive temperature. Thus, the role of Clf1p in DNA replication may be restricted to initiation, an inference that is in accord with the observation that mutants of the ORC complex also fail to show synthetic lethality or decreased viability when combined with the rad53, rad9, and mec1 checkpoint mutants (Dillin and Rine 1998).

An alternative explanation for our plasmid loss results is that Clf1p helps recruit or activate protein kinases that phosphorylate replication factors and thereby trigger the onset of DNA replication. Mutation of the S-phase cyclins CLB5 and CLB6, for example, substantially delays the onset of S phase (Donaldson et al. 1998) but to our knowledge has little effect on replication fidelity or plasmid loss rates. Thus, we are currently investigating the possible involvement of Clf1p in CLB-CDK-dependent steps in replication.

The discovery that a factor that is thought to play a role in pre-mRNA splicing might also be involved in DNA replication was surprising, although previous studies have linked the splicing factors Prp8p and Prp17/Cdc40p to cell-cycle progression in S phase. Reciprocal shift experiments with prp8 mutants revealed an execution point early in S phase and an arrest point at the G2/M transition, and prp17/cdc40 mutants entered S phase after a delay and arrest with 2C DNA (Shea et al. 1994; Boger-Nadjar et al. 1998). Although it is possible that the cell-cycle defects observed in splicing mutants result from a failure to splice a rate-limiting factor required for cell-cycle progression, the observation that clf1, prp8, and prp17/cdc40 mutants arrest within the first cell cycle after a shift to the restrictive temperature suggests a direct role in cell-cycle progression. Thus, splicing complexes have an additional heretofore unsuspected role in DNA replication, or, alternatively, complexes involved in splicing and DNA replication are distinct but share common subunits. As summarized in the Introduction, some TPR proteins are thought to serve as scaffolds for assembly of protein complexes while others serve as chaperones. If Clf1p is involved in multiple, distinct cellular processes, as now seems likely, it may serve as a loading factor or chaperone during the assembly of both pre-mRNA splicing complexes and replication initiation complexes. Factors known to help assemble replication initiation complexs in eukaryotes include Cdc6p, which is thought to load the MCM complex (Donovan et al. 1997; Tanaka et al. 1997; Perkins and Diffley 1998), and replication factor C, which loads proliferating cell nuclear antigen (Waga and Stillman 1998). It is noteworthy as well that the prokaryotic chaperones DnaK, DnaJ, and GrpE remove a factor that inhibits the activation of DNA helicase (Stillman 1994). It seems likely that chaperones will be found to play equivalent roles in eukaryotes.

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LITERATURE CITED


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