MOLECULAR AND CELLULAR BIOLOGY, Nov. 1986, p. 4099–4103
0270-7306/86/114099-05$02.00/0

Vol. 6, No. 11

Sequence Analysis of Temperature-Sensitive Mutations in the *Saccharomyces cerevisiae* Gene *CDC28*

ATTILA T. LÖRINCZ† AND STEVEN I. REED‡*

Biochemistry and Molecular Biology Section, Department of Biological Sciences, University of California, Santa Barbara, California 93105

Received 7 April 1986/Accepted 27 June 1986

Eleven independently isolated temperature-sensitive mutations in the cell division cycle gene *CDC28* were mapped with respect to the DNA sequence of the wild-type gene and then sequenced to determine the precise nature of each mutation. The set yielded six different point mutations, each of which predicts a single amino acid substitution in the *CDC28* product. The positions of the mutations did not correlate in any obvious way with observable biological characteristics of the mutant alleles. When the positions of substitutions were collated with a predicted secondary structural analysis of the *CDC28* protein kinase, they were found to correlate strongly with probable regions of structural transition.

Genetic analysis of division control in the budding yeast *Saccharomyces cerevisiae* has identified a number of genes which are required for cell cycle initiation (8, 17). DNA sequence analysis has revealed that one of these, *CDC28*, encodes a polypeptide which has significant primary structure homology with known protein kinases, including a number of oncogenes (11). We have recently demonstrated that the *CDC28* product does, in fact, have an associated protein kinase activity (19). Because expression of *CDC28* is required for progression through the cell cycle, genetic analysis must use conditional mutations. A large number of temperature-sensitive (*ts*) *cdc28* mutants have been isolated by in vivo and in vitro mutagenesis and subjected to preliminary characterization (16, 17; S. I. Reed, *in* G. M. Cooper, ed., *Viral and Cellular Oncogenes*, in press). Presumably, these mutant alleles encode protein kinases which are active at 23°C, the permissive temperature, but are inactive or less active at 36°C, the restrictive temperature. For the three temperature-sensitive alleles tested, thermolabile protein kinase activity has been demonstrated in vitro (19). Because they encode protein kinases capable of undergoing temperature-dependent functional transitions, these *ts* mutant alleles may be useful for relating protein kinase function to structure. We report here the DNA sequence of six point mutations conferring a *ts cdc28* phenotype and some possible protein structural implications of the predicted amino acid substitutions.

To facilitate the rapid sequencing of the mutant alleles, we first established the approximate locations of the mutations by DNA fragment-mediated gene conversion, as shown in Fig. 1a and b (20). Specific analytical fragments of wild-type DNA were prepared and used to transform mutant *S. cerevisiae* cells. Fragments spanning the mutant sites were able to recombine and rescue the temperature-sensitive mutant phenotype.

Single-stranded recombinant M13 phage DNA was prepared as previously described (13). Typically, 20 μg of each strand was annealed at 65°C in 40 μl of solution containing 500 mM NaCl, 1 mM EDTA, and 10 mM Tris hydrochloride, pH 8.0. After 2 h, the reaction mixtures were diluted with 35 μl of water, and S1 digestion of single-stranded DNA was initiated by addition of 20 μl of 5× S1 buffer (20 mM $ZnSO_4$, 1.25 M NaCl, 150 mM sodium acetate, pH 4.6) and 5 μl of S1 nuclease (Boehringer Mannheim, 1,000 U/μl). The reaction was allowed to proceed at 37°C for 30 min. DNA was then extracted with phenol-chloroform-isoamyl alcohol, precipitated with ethanol, and suspended in 15 μl of TE buffer (1 mM EDTA, 10 mM Tris hydrochloride, pH 8.0). Log-phase yeast cultures were transformed (9), spread on rich nutrient plates (YEPD), and incubated at 23°C for 16 h prior to incubation at 38°C. Rescuing fragments typically produced 20 to 200 colonies per 5 μg of DNA that were capable of growth at 38°C, while control fragments produced 0 to 2 colonies (presumably by spontaneous reversion of the mutant allele). Mutant yeast strains were derivatives of strains described previously (16, 17) which had been backcrossed to strain 381G (7) between 2 and 10 times. In all, 11 mutations resulting from in vivo mutagenesis by ethyl methanesulfonate (EMS) were analyzed.

Having defined the approximate locations of the mutations (to within 80 base pairs), relevant mutant chromosomal sequences were transferred into plasmid shuttle vectors by the gap repair method of Orr-Weaver et al. (15). For the purposes of this study, plasmid YRp7 (*CDC28.4*) (18), containing the wild-type gene and flanking sequences as well as the selectable marker *TRP1*, was used. Gapped plasmid DNA, repaired and recircularized in *cdc28* mutant yeast cells, was tested for the ability to rescue the corresponding temperature-sensitive mutation by retransformation. A negative result indicated that the mutant allele had been cloned. Mutant alleles were sequenced in M13 phage vectors by using the exonuclease Bal 31 to generate endpoints (11). Typical DNA sequencing data are shown in Fig. 1c.

Eleven temperature-sensitive alleles of *cdc28* which were originally derived by mutagenesis with EMS (16, 17) were mapped (Fig. 1a and b) and sequenced (Fig. 1c). All contained G-C to A-T transitions resulting in a single-codon change (Fig. 2). Note that 5 of 11 mutations analyzed are omitted from this summary as a result of redundancies within the set *cdc28-4* = *cdc28-6*; *cdc28-9* = *cdc28-16* = *cdc28-17* = *cdc28-19*; *cdc28-13* = *cdc28-18*, even though all mutants were isolated independently. The phenomenon of

* Corresponding author.
† Present address: Bethesda Research Laboratories, Gaithersburg, MD 20877.
‡ Present address: Department of Molecular Biology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

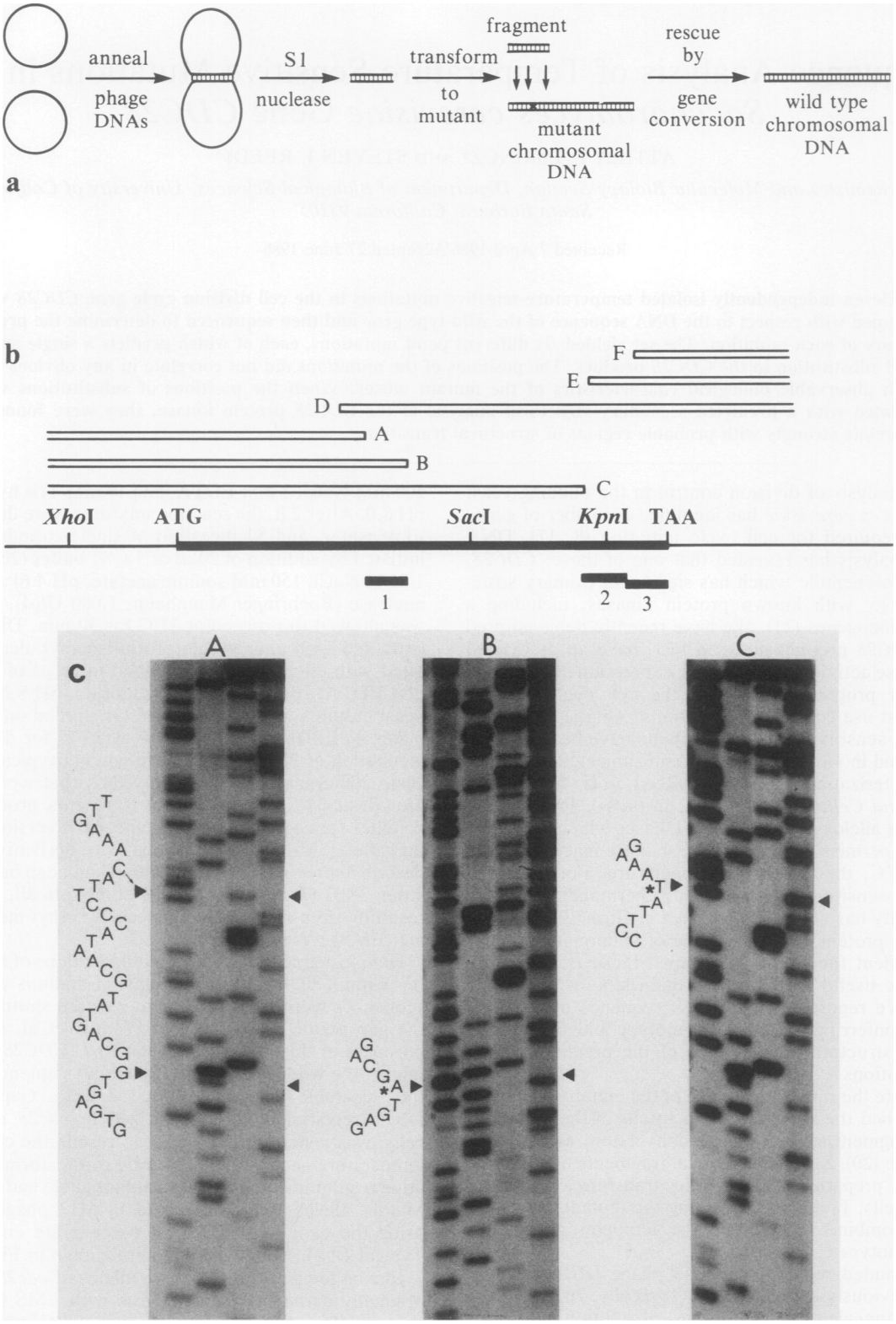

FIG. 1. Mapping and sequencing of *cdc28* mutant alleles. (a) To facilitate the rapid sequencing of the mutant alleles, we first established their approximate locations by DNA fragment-mediated gene conversion (20). For this procedure, double-stranded DNA fragments were generated by annealing opposite strands in appropriate combinations of M13 phage clones initially used to sequence the wild-type *CDC28* gene (11). Most of the endpoints for this set were generated by using exonuclease Bal 31. Double-stranded fragments were produced by digesting the heteroduplexed phage DNAs with S1 nuclease, and these fragments were in turn used to transform mutant yeast cells. Fragments spanning the mutant sites were able to rescue the temperature-sensitive mutant phenotype where gene conversion replaced mutant sequences with wild-type sequences. Such events were detected by incubating the transformation mixture at the restrictive temperature (38°C) and observing

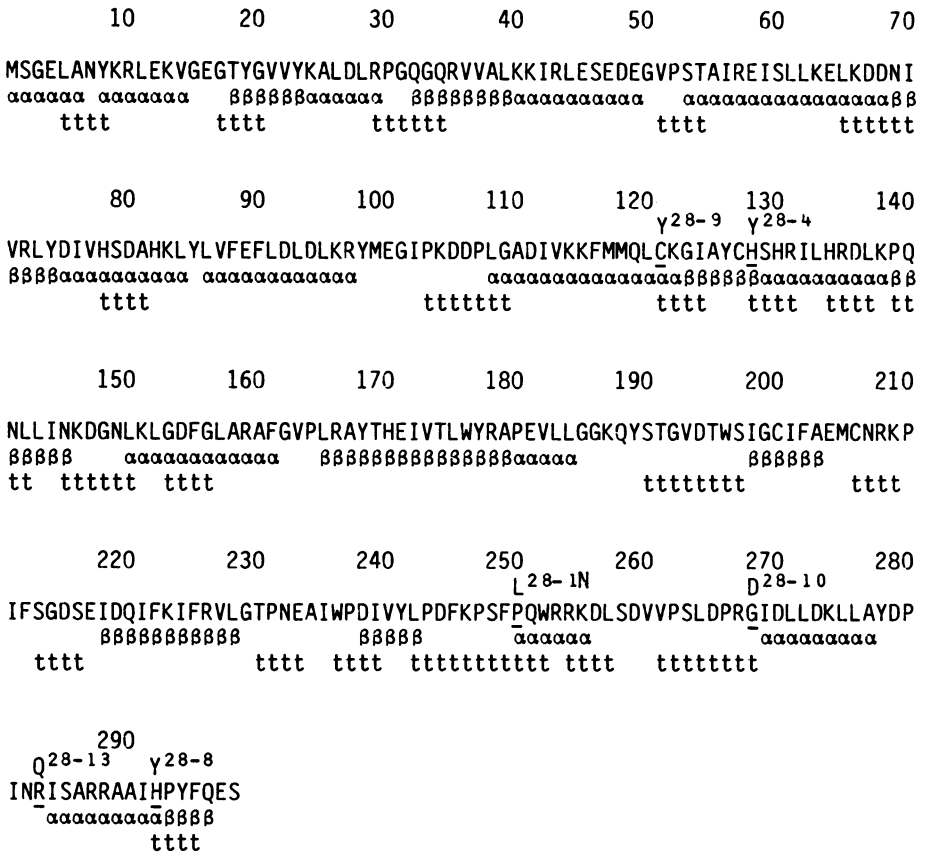

FIG. 2. Relationship of mutational substitutions to the primary and predicted secondary structures of the *CDC28* gene product. Single-letter codes for amino acid residues are used. Alphas indicate probable alpha-helical structures, and betas indicate probable beta structures according to the rules of Chou and Fasman (2, 3). t indicates regions where turns or reversals could occur (2, 3). Positions and substitutions corresponding to temperature-sensitive mutations are shown by underscoring and on the line above the wild-type sequence, respectively. Redundancies within the initial set of 11 mutations determined by sequencing are as follows: *cdc28-4* = *cdc28-6*; *cdc28-9* = *cdc28-16* = *cdc28-17* = *cdc28-19*; and *cdc28-13* = *cdc28-18*.

independent repetitive isolation of favored alleles (mutational hot spots) has been reported but is not understood (1, 4). Preliminary analysis of alleles isolated by in vitro mutagenesis suggests that the redundancy does not result from rigid constraints on substitutions that can yield *ts* mutations.

We wished to determine whether the distribution of the temperature-sensitive substitutions corresponded to any biological or predicted structural features of the *CDC28* protein. Mutagenesis by EMS (G-C to A-T transitions) can generate maximally only two substitutions at any one codon and minimally none (5). It is certain, therefore, that the number and variety of specific substitutions possible for each codon must in turn limit the number of positions available for the generation of temperature-sensitive mutations. With this caveat, as well as that concerning the small sample size and the possibility of mutagenic hot spots, it appears that temperature-sensitive proteins are generated primarily by substitution in two regions of the *CDC28* polypeptide (Fig. 2). One of these is relatively restricted and internal, whereas the other corresponds to the carboxy-terminal 50 residues. Preliminary mapping of a much larger set of temperature-sensitive mutations generated by in vitro mutagenesis of *CDC28* suggests that even though it is possible to isolate mutations throughout most of the coding region, these two subregions are clearly favored for the generation of *ts* alleles (S. Reed and B. Cheetham, unpub-

the appearance of wild-type colonies. (b) Schematic illustration of the *CDC28* coding region, showing the boundaries within which mutations were localized by marker rescue. Shown above are the endpoints of fragments used in rescue experiments. While fragment A could rescue no mutation, fragment B rescued mutations *cdc28-4*, *cdc28-6*, *cdc28-9*, *cdc28-16*, *cdc28-17*, and *cdc28-19*. The endpoints of these fragments therefore defined the boundaries of block 1. Similarly, the endpoints of fragments E and F defined the boundaries of block 2, within which *cdc28-1N* and *cdc28-10* were localized. Three mutations, *cdc28-8*, *cdc28-13*, and *cdc28-18*, were localized between the endpoint of fragment F and the carboxy terminus of the coding region (block 3). (c) The mutant alleles were sequenced by the deletion strategy described previously. In each case only a single base pair substitution was found in the target region. The sequence was determined for 400 bp flanking each mutation to exclude multiple substitutions. Shown are some dideoxy sequencing runs typical of the data obtained. Lanes (left to right for each panel) are A, G, C, and T. Panel A, Wild-type sequence in region corresponding approximately to codons 110 to 140; panel B, sequence from *cdc28-4* in same region (asterisk indicates mutated base); panel C, sequence from *cdc28-9* in same region (asterisk indicates mutated base). The sequence was read from the noncoding strand toward the 5′ end of the gene. The exact position and substitution corresponding to each mutation are shown in Fig. 2.

lished data). There was no obvious correlation between the positions of substitutions and the biological properties of the mutants. Differences in mutant cell morphology compared by using congenic strains appeared to relate more to such parameters as the ratio of gene product activity at the restrictive and permissive temperatures and the level of residual activity remaining at the restrictive temperature. For example, arrest at the restrictive temperature with a large fraction of budded non-G1-phase cells, a characteristic of the alleles *cdc28-1N* and *cdc28-4*, appeared to correlate with constitutively low activity, as defined by aberrant cell morphology, at the permissive temperature. Synchronous G1 arrest with virtually no budded cells, as observed for *cdc28-9* and *cdc28-13*, correlated with normal activity at the permissive temperature and rapid loss of activity at the restrictive temperature. Previous studies have indicated that mutations in both the central and carboxy-terminal regions affect protein kinase activity (19), although these experiments cannot discriminate between various aspects of catalysis, such as substrate binding and efficiency of phosphoryl transfer. It is noteworthy that none of the substitutions except for that corresponding to *cdc28-4* fell in a region or on a residue highly conserved among protein kinases (11). *cdc28-4* marks the amino-terminal boundary of a relatively conserved protein kinase consensus region (11). Of particular interest was the clustering of mutations near the carboxy terminus, a region known to be highly divergent in protein kinases. Yet a mutation as close as seven residues from the carboxy terminus appeared to be important for proper function of the protein. It is interesting, however, that antibodies directed against the extreme carboxy terminus of the v-*mos* oncogene product inhibit the protein kinase activity of this enzyme (12), suggesting a critical role for this region in an evolutionarily related molecule. Evaluating the significance of these observations will require more precise physiological and biochemical determinations on a larger set of mutant *cdc28* proteins.

A probable secondary structure, as predicted by the method of Chou and Fasman (2, 3) is shown in Fig. 2. Although the limitations of such procedures for precise prediction are generally accepted (3, 10), any strong correlation between the positions of temperature-sensitive substitutions and an aspect of predicted structure may imply a significant relationship between these positions and some aspect of the actual structure. The first question addressed was what, if any, predicted features correlated with substituted positions. Scrutiny of Fig. 2 indicates that the mutational substitutions were all positioned at what may be considered regions of structural transition, based on the Chou-Fasman algorithm. These are regions where the propensity for a defined structure changes, suggesting a transition from one structural form to another. None of the mutations was centrally embedded in a region predicted as an alpha-helix or a beta structure. For example, *cdc28-9* and *cdc28-4* placed substitutions at positions 121 and 128, respectively. These correspond to adjacent regions of alpha to beta and beta to alpha transition, respectively (Fig. 2). Every substituted position was within one residue of the end of a predicted alpha-helical segment (Fig. 2). The probability of such a distribution being generated by chance is <0.001. These data provide support for a relationship between the predicted structure and the true secondary structure, at least for this protein. Furthermore, secondary-structure analysis of the mutant proteins predicted a significant alteration of structure for each substitution (data not shown).

The constraints inherent in temperature-sensitive function, specifically a functional transition within a relatively narrow temperature range, may be consistent with the positioning of substitutions in regions of structural transition. The mutational substitutions may then affect the ability of adjacent units of structure (e.g., alpha helices or beta sheets) to be clearly defined at boundaries or to be packed appropriately relative to each other. Perhaps alternative conformational forms of the protein, highly unfavorable in the wild type, can be stabilized in the mutant forms at elevated temperature. Such transitions may be more readily reversible than gross alterations of folded structure resulting from substitutions in highly determined structural regions.

There is only one instance in which temperature-sensitive mutational analysis has been collated with the known three-dimensional structure of a protein (6). The observations of this study of phage T4 lysozyme concur only partially with ours. Of four *ts* mutations analyzed, two fell at the ends of alpha-helical segments and two others fell in internal portions of the same respective helices, suggesting that different criteria for thermolability may apply to different proteins. On the other hand, we have collated published data on a large number of temperature-sensitive *Escherichia coli lac* repressor mutations (14) with a secondary structure prediction of the protein (3) and have found, for the most part, that these mutations tend to cluster at predicted regions of structural transition, as is the case with the *CDC28* protein kinase.

This work was funded by Public Health Service grant RO1 GM-28005 from the National Institutes of Health and by National Science Foundation grant DCB84-02344 to S.I.R. S.I.R. was supported in part by American Cancer Society Faculty Research Award FRA-248.

Secondary structure analyses were performed with the aid of the BIONET structure routine. We thank Ralph Arlinghaus, Richard Lerner, and Peter Wright for critical readings of this manuscript.

LITERATURE CITED

1. **Benzer, S.** 1961. On the topography of the genetic fine structure. Proc. Natl. Acad. Sci. USA **47**:403–416.
2. **Chou, P. Y., and G. D. Fasman.** 1974. Prediction of protein conformation. Biochemistry **13**:222–245.
3. **Chou, P.Y., and G. D. Fasman.** 1978. Prediction of secondary structure of proteins from their amino acid sequence. Adv. Enzymol. **47**:45–147.
4. **Coulondre, C., and J. H. Miller.** 1977. Genetic studies of the *lac* repressor. IV. Mutagenic specificity of the *lacI* gene of *Escherichia coli*. J. Mol. Biol. **117**:577–606.
5. **Drake, J. W., and R. H. Baltz.** 1976. The biochemistry of mutagenesis. Annu. Rev. Biochem. **45**:11–38.
6. **Grutter, M. G., L. H. Weaver, T. M. Gray, and B. W. Matthews.** 1983. Structure, function and evolution of the lysozyme from bacteriophage T4, p. 356–360. *In* C. K. Mathews, E. M. Kutter, G. Mosig, and P. M. Berget (ed.), Bacteriophage T4. American Society for Microbiology, Washington, D.C.
7. **Hartwell, L. H.** 1980. Mutants of *Sacchromyces cerevisiae* unresponsive to cell division control by polypeptide mating pheromone. J. Cell Biol. **85**:811–822.
8. **Hartwell, L. H., J. Culotti, J. Pringle, and B. Reid.** 1974. Genetic control of the cell division cycle in yeast. Science **183**:46–51.
9. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
10. **Kabsch, W., and C. Sander.** 1984. On the use of sequence homologies to predict protein structure. Proc. Natl. Acad. Sci. USA **81**:1075–1078.
11. **Lörincz, A. T., and S. I. Reed.** 1984. Primary structure homology between the product of yeast division control gene *CDC28* and vertebrate oncogenes. Nature (London) **307**:183–185.

12. **Maxwell, S. A., and R. B. Arlinghaus.** 1985. Use of site-specific antibodies to perturb the serine kinase activity of Moloney murine sarcoma virus-encoded p37^{mos}. J. Virol. **55**:874–876.
13. **Messing, J., R. Crea, and P. A. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9**:309–321.
14. **Miller, J. H.** 1979. Genetic studies of the *lac* repressor. XI. On aspects of *lac* repressor structure suggested by genetic experiments. J. Mol. Biol. **131**:249–258.
15. **Orr-Weaver, T. L., J. W. Szostak, and R. J. Rothstein.** 1983. Genetic applications of yeast transformation with linear and gapped plasmids. Methods Enzymol. **101**:228–245.
16. **Piggott, J. R., R. Rai, and B. L. A. Carter.** 1982. A bifunctional gene product involved in two phases of the yeast cell cycle. Nature (London) **298**:391–393.
17. **Reed, S. I.** 1980. The selection of *S. cerevisiae* mutants defective in the start event of cell division. Genetics **95**:561–577.
18. **Reed, S. I., J. Ferguson, and J. C. Groppe.** 1982. Preliminary characterization of the transcriptional and translational products of the *Saccharomyces cerevisiae* cell division cycle gene *CDC28*. Mol. Cell. Biol. **2**:412–425.
19. **Reed, S. I., J. A. Hadwiger, and A. T. Lörincz.** 1985. Protein kinase activity associated with the product of the yeast cell division cycle gene *CDC28*. Proc. Natl. Acad. Sci. USA **82**:4055–4059.
20. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101**:202–211.