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Genetics

Dominant negative protein kinase mutations that confer a G_1 arrest phenotype

(cell division/division control/*Saccharomyces cerevisiae*/yeast)

MICHAEL D. MENDENHALL, HELENA E. RICHARDSON, AND STEVEN I. REED*

Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037

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ABSTRACT **The *CDC28* gene of *Saccharomyces cerevisiae* encodes a protein kinase that is required for passage through the G_1 phase of the cell cycle. We have used an inducible promoter fused to the *CDC28* coding sequence to isolate conditionally dominant mutant alleles of *CDC28*. Overexpression of these dominant alleles causes arrest in the G_1 phase of the cell cycle but permits the distinctive asymmetric growth that is characteristic of recessive temperature-sensitive *cdc28* mutants. The dominant alleles encode products with no detectable protein kinase activity, and their phenotypic effects can be suppressed by simultaneous overproduction of the wild-type protein. DNA sequence analysis showed that the mutant site in at least one of the dominant alleles is in a residue that is highly conserved among protein kinases. These properties are best understood if the dominant mutation results in the catalytic inactivation of the protein kinase but still allows the binding of another component needed for *CDC28* function. By this model, high levels of the mutant protein arrest cell division by denying the wild-type protein access to this other component. Suppressors that may encode this other component have been isolated on high-copy-number plasmids.**

In recent years much progress has been made in identifying the components that regulate entry into the eukaryotic cell division cycle, but little is known about how the individual elements interact. The budding yeast *Saccharomyces cerevisiae* is a useful system for studying such interactions because of its amenability to genetic manipulations. Moreover, many genes important for commitment to the yeast mitotic cell cycle have already been described, some of which have homology to the vertebrate oncogenes thought to be involved in growth control (1–4). One of these, *CDC28*, was originally identified in a screen of recessive, temperature-sensitive (*ts*) lethal mutations (5). The *ts* alleles of *cdc28* cause cells to arrest in an unbudded state at the restrictive temperature (5) while cellular growth continues, resulting in enlarged cells with a morphology similar to that seen when cells are treated with mating pheromone (6). Cells arrested at this point are still capable of conjugation (7), but once past the step defined by *cdc28* mutations, they are committed to the mitotic cell cycle. In recognition of its importance, Hartwell designated this point of the cell cycle as "Start" (8). Start is thought to be the point of the yeast cell cycle where environmental stimuli exert their control.

The product of *CDC28*, Cdc28, is a protein kinase (2, 9). It is part of a multiprotein complex that contains a 40-kilodalton substrate, p40, and possibly other factors (C. Wittenberg and S.I.R., unpublished data). Cdc28 activity is observed *in vitro* by monitoring the transfer of phosphate from [γ-^{32}P]ATP to p40 in immunoprecipitates. By using this assay, p40 phosphorylation has been detected only in actively cycling G_1 phase cells (10). Activity was lost in cells that have exited from the cell cycle (in response to nutrient limitation or pheromone exposure) or in cells that have completed G_1 and entered S phase. However, these two conditions are regulated differently because kinase activity was restored when extracts from non-G_1-phase cycling cells, but not extracts from stationary or pheromone-treated cells, were mixed with kinase-deficient *cdc28* mutant extracts prior to assay. Both the formation of the Cdc28 complex and its activation appear to be separately controlled (C. Wittenberg and S.I.R., unpublished data).

To understand the mechanism by which this control is mediated, it is necessary to identify the components with which the *CDC28* gene product interacts. One possible means of doing so might be to isolate extragenic suppressors of *ts cdc28* mutations, but this approach has resulted primarily in the isolation of intragenic revertants (unpublished data). In this paper we describe the isolation of a type of *CDC28* allele that may enable us to identify and isolate genes whose products interact with the *CDC28* gene product. These alleles are lethal when highly expressed, conferring a G_1 arrest phenotype similar to that conferred by loss-of-function mutations. The simplest explanation for the properties of these alleles is that the mutant protein nonproductively binds a component needed for *CDC28* function, blocking the coexisting wild-type protein's access to this component. The resulting inability to form an active protein kinase complex would then prevent the cell from completing the Start event and initiating a new cell cycle.

MATERIALS AND METHODS

Strains and Media. The following yeast strains were used: D4 (*MATα ade1 cdc28-4 his3 leu2 trp1*), D13 (*MATα ade1 cdc28-13 his3 leu2 trp1*), D63 (*MATα ade1 cdc28-63 his3 leu2 trp1*), J16D (*MATa his3 leu2 trp1 ura3* [cir^0]; obtained from M. Jayaram), JAHy32 (*MATa/MATα his3Δ/his3Δ leu2-3,112/leu2-3,112 trp1/trp1 ura3-52/ura3-52* [cir$^+$]), yh102 (YIpGAL1p::CDC28-dn1 transformant of JAHy32), MDMy337 (*MATa ade1 ade2 cdc7-1 gal1 his7 lys2 tyr1 ura1*), MDMy356 (YCpGAL1p::CDC28 transformant of J16D), MDMy357 (YIpGAL1p::CDC28-dn1 transformant of J16D), MDMy358 (YIpGAL1p::cdc28-4 transformant of J16D), MDMy359 (YCpGAL1p::CDC28 transformant of D4), MDMy364 (YIpGAL1p::cdc28-63 transformant of J16D), and MDMy368 (YCpGAL1p::CDC28-dn1 transformant of D4). Above strains carrying chromosomally integrated copies of YIpGAL1p::CDC28 and its allelic derivatives were constructed by digesting the plasmid in the *URA3* region with *Sma* I and transforming J16D (11). Yeast transformations were by the method of Ito *et al.* (12). YEP is 1% yeast extract/2% Bacto-peptone (Difco). Noninducing medium is the appropri-

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Abbreviations: *ts*, temperature sensitive mutation; *dn*, dominant negative allele.

*To whom reprint requests should be addressed.

ate selective medium (13) supplemented with 1.5% glycerol and 1.5% ethanol. For induction of the *GAL1* promoter, 2% galactose was added.

Plasmid Constructions. YCpGAL1p::CDC28 was constructed as follows. The *Sal* I–*Xho* I fragment carrying the *LEU2* gene was cloned from YEp13 (14) into the *Sal* I site of pBM258 to generate YCpG1 (C. Wittenberg and S.I.R., unpublished data). pBM258 (obtained from M. Johnston) is identical to pBM150 described by Johnston and Davis (15). The *Bam*HI–*Bgl* II fragment carrying the *CDC28* gene from YRp7'[CDC28.4(BamHI)] (16) was cloned into the *Bam*HI site of YCpG1 to generate YCpGAL1p::CDC28 (Fig. 1). The *Bam*HI site in YRp7'[CDC28.4(BamHI)] had been introduced by oligonucleotide mutagenesis and is 10 base pairs upstream from the translation initiation codon of *CDC28*. This construction puts the *CDC28* gene under the control of the *GAL1* promoter (designated GAL1p in constructs). Derivatives containing *ts* alleles were constructed from YCpG1 in a similar fashion. YIpGAL1p::CDC28 (and its mutant derivatives) was constructed by cloning the *Eco*RI–*Xba* I fragment containing the *GAL1p::CDC28* fusion from YCpGAL1p::CDC28 (or its mutant derivatives) into the multiple cloning site of pUC19-URA3 (obtained from J. Nickoloff, Research Institute of Scripps Clinic) (Fig. 1). The *Sma* I site in the multiple cloning site of pUC19-URA3 has been eliminated.

Preparation of a *CDC28* Mutant Library. YCpGAL1p::CDC28 was mutagenized by treatment with hydroxylamine by the following modification of the procedure of C. Franklyn and N. Lee (personal communication). Ten micrograms of plasmid DNA in 50 μl of 1 M potassium phosphate (pH 6.0) was treated with 50 μl of 1 M hydroxylamine (freshly dissolved) at 70°C for 1 hr. This mixture was then desalted over a Sepharose CL-6B spin column and used to transform *Escherichia coli* strain BD1528 (*thyA met⁻ nadBF ung⁻ gal⁻ supE supF hsdR⁻ hsdM⁺*) (17). At least 5×10^4 bacterial transformants were pooled. The library was amplified by growth in LB medium containing ampicillin, and plasmid DNA was isolated (18) and used to transform *S. cerevisiae* strain J16D.

Other Methods. The DNA sequences of *CDC28-dn1* and *CDC28-dn2* were determined by the method of Sanger *et al.* (19); *dn* signifies dominant negative. Short oligonucleotides corresponding to portions of the *CDC28* gene spaced every 150 base pairs were used as primers to sequence the entire coding region for both alleles. The primers were synthesized on an Applied Biosystems (Foster City, CA) 380B DNA synthesizer. Assays of Cdc28 protein kinase activity and relative quantitation of the amounts of Cdc28 protein by immunoblot analysis were performed as described by Mendenhall *et al.* (10). Cell number and budding index were determined by counting with a hemacytometer. DNA content was determined by flow cytometric analysis of propidium iodide-stained cells (20) on a Becton–Dickinson FACS IV instrument. Relative mean cell size was determined on the FACS IV (in arbitrary units) by analysis of the low angle (forward) light scatter of cells with 1 centimorgan DNA content.

RESULTS

Isolation and DNA Sequence. To provide controlled expression of the *CDC28* mutant alleles, the plasmid YCpGAL1p::CDC28 carrying the *CDC28* coding region fused to the *GAL1* promoter was constructed (Fig. 1). The *GAL1* promoter is dually regulated, being repressed by glucose and induced by galactose. The use of this construct allowed us to isolate and propagate potentially dominant lethal alleles of *CDC28*. YCpGAL1p::CDC28 was treated with hydroxylamine to generate a mutant *CDC28* library. A *CDC28⁺* yeast strain, J16D, was transformed with this library, and the transformants were allowed to grow on a noninducing selective medium. The colonies then were replica-plated to selective medium containing galactose and screened for colonies that failed to grow. Four mutants were obtained after screening ≈2000 transformants.

Microscopic examination of the galactose-sensitive mutants after the shift to the inducing medium revealed that they arrested predominantly as unbudded cells with a morphology characteristic of *ts cdc28* mutants and of arrest at Start in general (Fig. 2). To prove that the phenotype was conferred by the plasmid-born *CDC28* allele, plasmid DNA was isolated from two of the transformants that were unable to grow on medium containing galactose and was used to retransform yeast cells. In both cases, the transformants were unable to grow when shifted to galactose medium, indicating that the plasmids did confer the galactose sensitivity. The sites of the mutations were further localized by cloning the *Eco*RI–*Xba* I fragment (Fig. 1) carrying the *GAL1* promoter–*CDC28* fusion from each of the plasmids into an integrating vector (Fig. 1). Transformants carrying either of these constructions were galactose sensitive, indicating that the mutations are most likely in the *CDC28* gene. We have called these alleles *CDC28-dn1* and *CDC28-dn2*.

The precise sites of the lesions in these two mutants were determined by sequencing the entire *CDC28* coding sequence of both plasmids (Fig. 3). In each case only a single nucleotide change resulting in a single amino acid substitution was found. *CDC28-dn1* had asparagine at amino acid position 154 instead of the wild-type aspartate, and *CDC28-dn2* had serine at position 164 instead of wild-type proline. The aspartate at 154 is part of the Asp-Phe-Gly sequence that is found in almost all known eukaryotic protein kinases (24). The proline at 164 is not as highly conserved but is found in the functionally homologous protein kinases from *Schizosaccharomyces pombe* (22, 25) and humans (23) and in Kin28, a structurally related protein kinase from *S. cerevisiae* (26). As

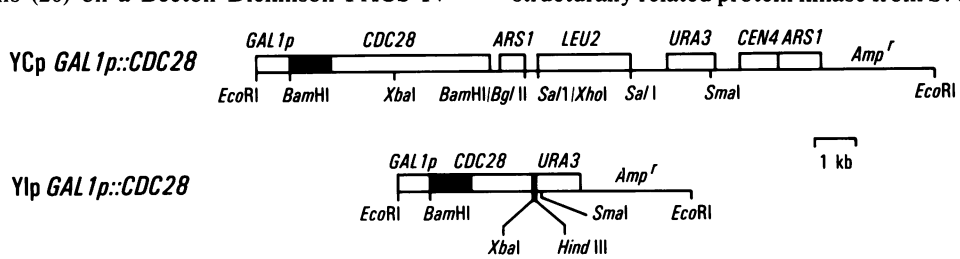

FIG. 1. Structures of the plasmids used. The shaded area indicates the position and extent of the *CDC28* coding region. *GAL1p* is the transcription control region 5' to the *GAL1* gene, *ARS1* is a sequence that allows the plasmid to be autonomously replicated in yeast, *LEU2* and *URA3* are yeast-selectable markers, *CEN4* is a centromeric sequence that stabilizes the plasmid in single copy number, and *Amp^r* encodes β-lactamase, which allows the plasmid to be selected in *Escherichia coli*. Pertinent restriction sites are indicated below the line. The YCp plasmids replicate autonomously in yeast. They are stable and present in approximately one copy per cell (21). The YIp plasmids cannot replicate in yeast. Transformation with YIp plasmids occurs by integration into the yeast genome.

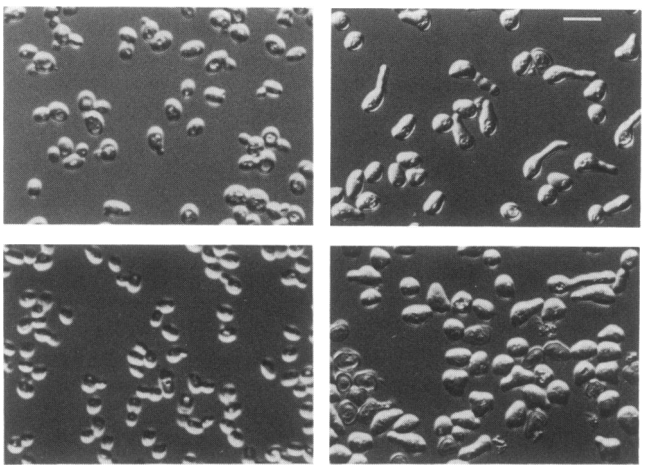

FIG. 2. Morphology of cells carrying the *CDC28-dn1* mutation. (*Upper*) MDMy357 strain (YIpGAL1p::CDC28-dn1). (*Lower*) D13 strain (*cdc28-13*^{ts}). (*Left*) Cells were grown under permissive conditions (23°C in yeast extract/peptone containing 1.5% glycerol and 1.5% ethanol). (*Right*) Cells were grown for 6 hr after adding galactose (2% final concentration) and increasing the temperature to 38°C. Photomicrographs were taken with a Zeiss Axiophot photomicroscope using differential interference contrast (Nomarski) optics. (Bar in *Upper Right* = 10 μm.)

the properties of both *dn* alleles are similar, we will limit further discussion to the *Cdc28-dn1* allele.

Physiology. As we have shown (Fig. 2), overexpression of the *CDC28-dn1* allele results in a morphology similar to that of recessive *ts cdc28* mutants. We wished to examine whether the similarity extended to other phenotypes characteristic of the previously isolated *cdc28* alleles. When shifted to 2% galactose, a culture of MDMy357 (YIpGAL1p::CDC28-dn1; *CDC28*$^+$) arrested within one cell cycle and accumulated predominantly as unbudded cells (Fig. 2). Flow cytometric analysis indicated that 90% of the arrested cells had a single haploid complement of DNA, indicating arrest in G_1, and that cell size continued to increase (data not shown). When shifted to the restrictive temperature, a control strain bearing the *ts* allele *cdc28-13* behaved similarly (Fig. 2 and data not shown). A strain carrying YCpGAL1p::CDC28 (*CDC28*$^+$ under galactose control) displayed a momentary lag after the shift in temperature and carbon source but recovered and resumed normal growth. Like other Start mutants, and unlike most other *cdc* mutants (7), the *dn* alleles mate efficiently after arrest. After 3 hr of galactose induction, MDMy357 produced twice as many diploid colonies as did a wild-type control in quantitative filter matings (7). By comparison, a non-Start *cdc7* mutant arrested at the restrictive temperature for 3 hr mated 1/8000th as efficiently as wild type did (data not shown). In all their basic properties, therefore, the *dn* alleles resemble the previously isolated *ts* alleles.

Genetic dominance is often explained in terms of gains of functions (neomorphs) or increases in activity (hypermorphs). However, dominant phenotypes produced by these mechanisms are usually distinct from and are often the converse of the phenotypes of recessive mutations in the same gene. Because of the similarity of the phenotypes of the *dn* and *ts cdc28* mutations, we thought that the *dn* mutations were probably not neomorphs or hypermorphs. Instead, the properties of the *dn* mutations are best explained if the mutant protein were enzymatically inactive but still able to bind another component required for Cdc28 function. The phenotype resulting from overexpression of the *dn* mutation would then be due to the sequestration of this other component and the resulting inability of the wild-type protein to form an active enzymatic complex. If this explanation is correct, then overexpression of CDC28$^+$ should reverse the effects of overexpression of *CDC28-dn1*. To test this prediction, we transformed MDMy357 (YIpGAL1p::CDC28-dn1; *CDC28*$^+$) with YCpGAL1p::CDC28. When shifted to galactose, these cells continued to divide (Fig. 4). We obtained the same result when *CDC28*$^+$ was expressed from the very active *PGK1* promoter (27) in a strain bearing YCpGAL1p::CDC28-dn1. The ability of the overexpressed wild-type protein to rescue the defect caused by overexpression of CDC28-dn1 indicates that the CDC28-dn1 mutation is not a neomorph or a hypermorph. It also indicates that the defect is not simply due to the presence of large amounts of Cdc28 protein in the cell.

Protein Kinase Activity. We next tested whether Cdc28-dn1 and Cdc28-dn2 were catalytically inactive. To assay the activity of the *dn* alleles without interference from the

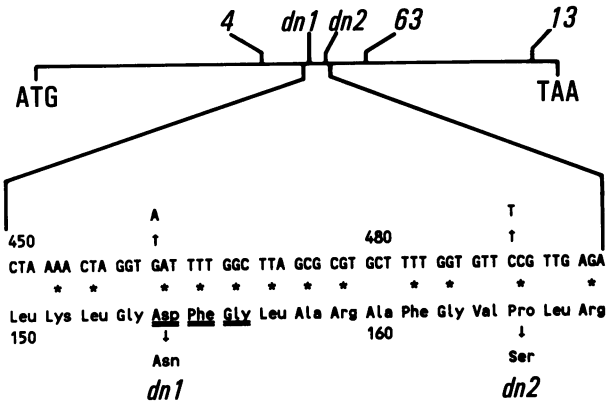

FIG. 3. DNA sequence of *CDC28-dn1* and *CDC28-dn2*. (*Upper*) Schematic representation of the coding portion of the *CDC28* gene (amino terminus at left). The sites of mutant alleles used in this study are indicated (*4* = *cdc28-4*, *dn1* = *CDC28-dn1*, *dn2* = *CDC28-dn2*, *63* = *cdc28-63*, and *13* = *cdc28-13*). (*Lower*) The region containing the *CDC28-dn1* and *CDC28-dn2* mutant alleles is expanded. The DNA (top line) and corresponding amino acid (bottom line) sequence of this region in the wild-type gene is shown. The mutant sites are indicated by the arrows. Asterisks indicate amino acids also present in *cdc2*$^+$ of *Schizosaccharomyces pombe* (22) and *CDC2*s of human (23). Double underlining indicates residues that are generally conserved among all protein kinases.

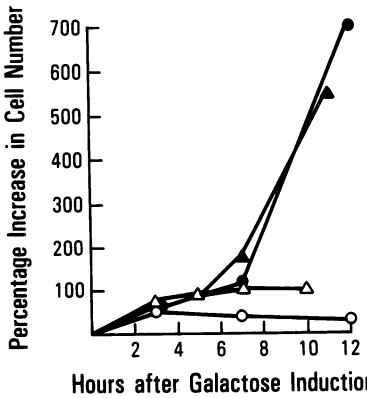

FIG. 4. Overexpression of *CDC28*$^+$ rescues overexpression of *CDC28-dn1*. The percentage increase in cell number was determined by counting on a hemacytometer at intervals after addition of 2% galactose to the media. Cells were pregrown in noninducing selective media. Strains used were as follows: MDMy357 (YIpGAL1p::CDC28-dn1; *CDC28*$^+$) (○), YCpGAL1p::CDC28 transformant of MDMy357 (●), YCpGAL1p::CDC28-dn1 transformant of J16D (*CDC28*$^+$) (△), and same as the last-named strain, except a *PGK1* promoter/*CDC28* fusion had been previously integrated at the *CDC28* chromosomal site (▲).

wild-type gene product, YCpGAL1p::CDC28-dn1 and YCpGAL1p::CDC28-dn2 were transformed into a *cdc28-4*-bearing strain. The *cdc28-4* strain has no measurable protein kinase activity *in vitro* (9) and, therefore, can serve as a neutral background in which the activity of other *cdc28* mutants can be measured. A control strain in which the *cdc28-4* strain carried YCpGAL1p::CDC28 was also constructed. The protein kinase activities of logarithmic cultures were determined at 30-min intervals after induction with galactose (Fig. 5). Activity appeared within 1 hr in the strain carrying the wild-type fusion but was never seen in either of the strains carrying the *dn* alleles (data for *CDC28-dn2* not shown). The quantity of Cdc28 protein was monitored at each time point by immunoblot analysis. Although the increase in Cdc28 protein could clearly be seen in these experiments (data not shown), we made use of the *cdc28-63* allele (B. Cheetham and S.I. Reed, unpublished data) to demonstrate the production of Cdc28 protein at each time point more clearly. This *ts* mutation produces a protein that migrates more slowly than the wild-type protein on $NaDodSO_4$/PAGE gels (C. Jones and S.I.R., unpublished data). The YIpGAL1p::CDC28-dn1 fusion was integratively transformed into D63 (*cdc28-63*), and extracts were made at 30-min intervals after galactose induction. The immunoblot in Fig. 5 clearly shows the rapid appearance of the Cdc28-dn1 protein and the invariant steady-state levels of Cdc28-63 protein under these conditions. These results indicate that the proteins encoded by the *CDC28-dn* alleles are enzymatically inactive, but we cannot rule out the possibility that they are active *in vivo* and labile *in vitro* as is the case with the product of the *cdc28-4* allele.

Since the *CDC28-dn* mutations are dominant, we examined the effect of induction of *CDC28-dn1* on the wild-type Cdc28 protein kinase activity. Most of the *in vitro* protein kinase activity in MDMy357 was lost within 90 min of galactose induction (Fig. 5). The activity loss is not simply a consequence of arrest at Start, since the Cdc28 protein kinase activity is still detected in extracts from cells arrested at the restrictive conditions in *cdc28*, *cdc37*, and *cdc39 ts* Start mutants when assayed at 23°C *in vitro* (unpublished data). The Cdc28 protein kinase activity loss due to galactose induction of the *CDC28-dn1* mutation is specific to the *dn* alleles, as overproduction of either the *cdc28-4* or the *cdc28-63* gene product does not affect the wild-type protein kinase activity (Fig. 5, data for *cdc28-63* not shown) and, in fact, does not produce any noticeable phenotypic alterations. The inability of overproduced ts mutant Cdc28 protein to affect the protein kinase assay is surprising because the antibody is limiting relative to antigen in these immunoassays, and at the later time points, the quantity of Cdc28 protein vastly exceeds the binding capacity of the antibody. We had expected the activity to decrease when the *ts* alleles were overexpressed because the wild-type protein would be competing for the limited amount of antibody with larger quantities of the mutant Cdc28 protein. The antibody used was raised against a peptide corresponding to the 10 amino-terminal residues of Cdc28 (10), and none of the *CDC28* alleles used have mutations in this region (ref. 28; K.-Y. Jahng and S.I.R., unpublished data). The anti-Cdc28 amino terminus antibody is capable of recognizing the Cdc28-63 protein, since protein kinase activity is detectable in this mutant at 23°C (unpublished data). This implies that either a small amount of wild-type protein is able to rescue the defect in the *ts* mutations or the wild-type protein can outcompete the mutant protein for formation of the active protein kinase complex, and it is only this complex that is being recognized by the antibody. In either case, the *dn* alleles behave differently.

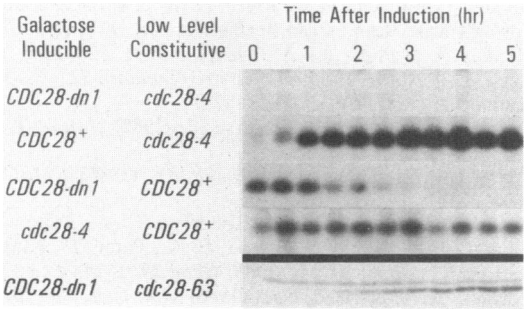

FIG. 5. Protein kinase activity of mutants carrying *CDC28-dn1*. The top four lines are autoradiograms of $NaDodSO_4$/PAGE gels displaying p40 phosphorylation activity at the times noted after galactose induction of strains carrying the *CDC28* alleles indicated on the left. The bottom line is an immunoblot displaying the concentration of Cdc28 protein at the times indicated. The upper band in this line is the product of the *cdc28-63* allele produced by its normal promoter; the lower band in this line (not seen at 0 hr) is the product of *GAL1p::CDC28-dn1*. Each strain used carries two alleles of *CDC28*. Alleles in the column labeled "Galactose Inducible" are under the control of the *GAL1* promoter. The alleles in the column labeled "Low Level Constitutive" are controlled by the wild-type promoter. The strains used are (from top to bottom) MDMy368, MDMy359, MDMy357, MDMy358, and MDMy364. See the legend to Fig. 1 for complete genotypes. Cells were grown in noninducing defined media and induced with 2% galactose. Under these conditions, the doubling time of the cultures was between 6 and 8 hr.

Suppressors. If the phenotypic effects of the *dn* alleles are due to their ability to sequester a component required for Cdc28 function, it may be possible to suppress the *dn* mutants by overproducing this other component. To obtain such suppressors MDMy357 (YIpGAL1p::CDC28-dn1 [cir^0] was transformed with a yeast genomic library constructed from the high copy YEp13 vector (29). Leu^+ transformants, isolated on galactose-containing medium, were tested for growth on galactose medium. Of ≈20,000 Leu^+ transformants scored, only 20 contained plasmids that allowed growth on galactose-containing medium after retransformation. Since YEP13 transformants of [cir^0] strains are very heterogeneous with respect to plasmid copy number (30), the plasmids were further analyzed after transformation into a [cir^+] strain containing YIpGAL1p::CDC28-dn1 (yh102). In this strain, only two of the plasmids resulted in galactose-insensitive transformants. This is probably due to the absence of the subpopulation of cells with an extremely high copy number that is found in the [cir^0] transformants and reflects more efficient suppression by these two plasmids. Restriction site analysis revealed that one of these plasmids carried $CDC28^+$ while the other had a 7-kb insert with a distinct restriction map.

Preliminary analyses (to be published elsewhere) indicate that the latter suppressing plasmid does not suppress at least one *ts* allele of *cdc28* (*cdc28-13*) and does not decrease the amount of Cdc28-dn1 protein produced from the *GAL1* promoter or increase the amount of $Cdc28^+$ protein produced from the wild-type promoter. Therefore, this latter plasmid is a candidate for a gene encoding the factor that Cdc28-dn1 apparently sequesters.

DISCUSSION

By using the *GAL1* promoter to provide controlled expression of the Cdc28 protein kinase gene, we have been able to isolate conditionally *dn* alleles of *CDC28*. When highly expressed, these alleles confer a phenotype similar to that of recessive *ts cdc28* mutations, but unlike the previously described mutations, the new alleles of *CDC28*, *CDC28-dn1*, and *CDC28-dn2* express their phenotype in a wild-type background. The dominance could be explained by (*i*) an acquisition of new properties (neomorphism or hypermorph-

ism) by the *dn* alleles, (*ii*) a reduction in the amount of Cdc28$^+$ induced by overproduction of the Cdc28-dn protein, (*iii*) a poison subunit effect analogous to the *lacI*$^{-d}$ mutations (reviewed in ref. 31), or (*iv*) sequestration by Cdc28-dn of a factor required for Cdc28 function.

Since simultaneous overexpression of the wild-type allele counteracted the effects of the mutant allele, neo- or hypermorphism is unlikely. The lack of *Cdc28-dn1*-encoded protein kinase activity in the *in vitro* assay is also consistent with this interpretation. The possibility that overproduction of the inactive Cdc28-dn protein results in the loss of the wild-type product because of induction of proteolysis or to feedback inhibition of transcription or translation was shown to be untenable through the use of the *cdc28-63* allele. As we show in Fig. 5, the level of product made from the wild-type promoter and maintained in the cell is uninfluenced by the greater amounts of protein made in response to galactose induction of *CDC28-dn1*. Although more difficult to dismiss, the possibility that Cdc28-dn1 acts as a poison subunit seems unlikely because sequences other than *CDC28* can suppress *CDC28-dn1* on multicopy plasmids.

We propose that the sites that have been mutated in *CDC28-dn1* and CDC28-dn2 are essential for catalysis but have little or no role in binding other components. Both mutations are in a region with an arrangement of amino acids that is highly conserved among eukaryotic protein kinases. This conservation is likely to be a reflection of the involvement of this region in catalysis and, therefore, less likely to be involved in specific recognition of substrates or regulatory components. Therefore, the most plausible explanation for the phenotypic behavior of these mutations is that, when overproduced, the mutant gene product outcompetes the wild-type protein for a factor needed for *CDC28* function. Since the *dn* gene product is enzymatically inactive, the complex that is formed is nonproductive, and the Start event carried out by *CDC28* cannot be completed. The finding that overexpression of wild-type Cdc28 can rescue cells overexpressing the *dn* mutations supports this aspect of competition between the wild-type and mutant alleles.

In addition to their use in probing the control of G_1 in *Saccharomyces*, these alleles are potentially useful for the study of cell-cycle control in genetically intractable systems. The *CDC28* gene is evolutionarily highly conserved. It has been shown by transformation that functionally homologous protein kinases exist in the human (23) and in the fission yeast *Schizosaccharomyces pombe* (25). All three protein kinases have similar sequences (2, 22, 23). Since both of the sites mutated in the *dn* alleles are conserved in all three genes, the analogous mutations could be constructed by site-specific mutagenesis in the human gene and then expressed in cultured cells. More generally, since the site that is altered in *CDC28-dn1* is highly conserved among protein kinases in general, similar mutations could be made in almost any protein kinase. Such alleles may be as useful for probing protein kinase function as the dominant GTPase-deficient mutations (32) have been for probing the function of GTP binding proteins.

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