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Saccharomyces cerevisiae cdc15 Mutants Arrested at a Late Stage in Anaphase Are Rescued by *Xenopus* cDNAs Encoding N-*ras* or a Protein with β-Transducin Repeats†

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We have constructed a *Xenopus* oocyte cDNA library in a *Saccharomyces cerevisiae* expression vector and used this library to isolate genes that can function in yeast cells to suppress the temperature sensitivity defect of the *cdc15* mutation. Two maternally expressed *Xenopus* cDNAs which fulfill these conditions have been isolated. One of these clones encodes *Xenopus* N-*ras*. In contrast to the yeast *RAS* genes, *Xenopus* N-*ras* rescues the *cdc15* mutation. Moreover, overexpression of *Xenopus* N-*ras* in *S. cerevisiae* does not activate the RAS-cyclic AMP (cAMP) pathway; rather, it results in decreased levels of intracellular cAMP in both mutant *cdc15* and wild-type cells. Furthermore, we show that lowering cAMP levels is sufficient to allow cells with a nonfunctional Cdc15 protein to complete the mitotic cycle. These results suggest that a key step of the cell cycle is dependent upon a phosphorylation event catalyzed by cAMP-dependent protein kinase. The second clone, β*TrCP* (β-transducin repeat-containing protein), encodes a protein of 518 amino acids that shows significant homology to the β subunits of G proteins in its C-terminal half. In this region, βTrcp is composed of seven β-transducin repeats. β*TrCP* is not a functional homolog of *S. cerevisiae CDC20*, a cell cycle gene that also contains β-transducin repeats and suppresses the *cdc15* mutation.

The molecular events that control entry into the cell cycle (G_1/S transition) and entry into mitosis (G_2/M transition) have been subjected to intensive investigation in recent years. Major breakthroughs in these studies were the discoveries that commitment to a new round of cell division occurs at a point termed START (34) and that nuclear division is brought about by the rapid activation of maturation promoting factor (MPF) (59). In order to pass START, yeast cells must grow to a critical size and monitor nutrient availability. A second control point, mediated by MPF, acts in late G_2 and determines when mitosis is initiated. MPF was first purified from *Xenopus* eggs and was shown to consist of two subunits: a 34-kDa serine/threonine kinase and a 45-kDa B-type cyclin (reviewed in references 48 and 67). The 34-kDa catalytic subunit has been subsequently identified as the *Xenopus* homolog of the yeast cdc2/Cdc28 kinase and of the mammalian growth-associated histone H1 kinase (44). Genetic analysis of both budding and fission yeasts has revealed that the very same kinase is involved in the regulation of START (63, 72, 90). While in *Saccharomyces cerevisiae* the cdc2/Cdc28 kinase controls both G_1/S and G_2/M transitions, in higher eukaryotes each transition is regulated by structurally related but functionally different kinases (23). These findings place the *cdc2/CDC28* gene family as the key regulator of the mitotic cell cycle.

The activity of the cdc2/Cdc28 kinase oscillates during the cell cycle as a result of variations in its state of phosphorylation and its association with other proteins, including cyclins (reviewed in references 48, 62, 67). The G_1/S function requires association with the G_1 cyclins (71) and the G_2/M function requires the B-type cyclins (27, 90). Activation of the kinase at the G_2/M boundary correlates with entry into mitosis, and inactivation is a prerequisite to exit from mitosis (62). Although it has been shown that degradation of the cyclin B subunit is essential to reduce cdc2 activity (27, 56), the process by which the kinase is inactivated is not well understood (62). The *S. cerevisiae* cell cycle gene *CDC15* is required for the completion of nuclear division, but its precise function is unknown (17, 81). Upon shift to the restrictive temperature, mutant *cdc15* cells arrest at a point late in anaphase as single budded, abnormally elongated, uninucleate cells with properly segregated chromosomes, complete spindles, and high levels of cdc2/Cdc28 kinase activity (55). To gain insight into the role played by *CDC15* and by other genes in the inactivation of the kinase and the coordination of signals that mediate exit from mitosis, we have looked for high-dosage suppressors of the temperature-sensitive phenotype of the *cdc15* mutation. This genetic strategy has been widely used both in fission and in budding yeasts to isolate genes encoding proteins that operate in the same pathway or interact in a complex. Given the conservation of basic cell cycle machinery among eukaryotes, we have assumed this type of analysis could also be performed across species boundaries. Recently, the complementation approach taken by Lee and Nurse (45) to isolate the human *cdc2* gene has been used successfully to isolate functional homologs of the G_1 cyclins from higher eukaryotes (43, 46, 47, 100), and the suppressor approach has led to the isolation of a new member of the human *cdc2* gene family (22, 61). We have chosen the genetic pool of an embryonic system to search for suppressors of the budding yeast *cdc15* mutation. *Xenopus* oocytes are particularly well suited for this purpose, because they contain the mRNA sequences for the proteins needed to regulate the various stages of cell cycle arrest and release from arrest which characterize oogenesis, oocyte maturation, fertilization, and cleavage stages. Since oocyte maturation and early embryonic development (up to the midblastula transition) occur in the absence of de novo transcription, the functions which so tightly control cell cycle arrest in meiotic prophase I at the full-grown oocyte

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† We dedicate this work to our colleague Mark B. Dworkin, who died of cancer on 30 April 1992.

TABLE 1. Yeast strains used in this study

Strain[a]	Genotype
WS165-2B	*MATα ade2 his3 leu2 lys1 trp1 ura3*
WS167-25	*MATα ade2 cdc15-2 his3 leu2 lys1 trp1 ura3*
WS183[b]	*MATα ade2 cdc15 his3 leu2 lys1 ras2::LEU2 trp1 ura3*
K838[c]	*MATa ade2 cdc15-2 his3 leu2 trp1 ura3*
K2773[c]	*MATα cdc20 his3 leu2 trp1 ura3*
S228C[d]	*MATα SUC2 mal mel gal2 CUP1*

[a] All strains, unless indicated otherwise, are from this work.
[b] Isogenic to WS167-25, except *ras2*.
[c] Source: Kim Nasmyth.
[d] Source: Yeast Genetic Stock Center, Berkeley, Calif.

stage and metaphase II in the unfertilized egg, as well as the rapid synchronous divisions of the cleaving embryo, must be stored in the form of maternal mRNA in the oocyte (42). Thus, an expression library from *Xenopus* oocyte cDNA provides a unique gene pool from which to identify regulatory sequences through complementation of *S. cerevisiae* cell cycle mutations. Furthermore, combination of genetic analysis in *S. cerevisiae* with the biochemical dissection of the cell cycle in *Xenopus* eggs and early embryos has led to significant advances in the understanding of the cell cycle. In this report we describe the identification of two *Xenopus* cDNAs, N-*ras* and β*TrCP*, and discuss the likely involvement of the RAS-cyclic AMP (cAMP) pathway in the completion of the cell cycle.

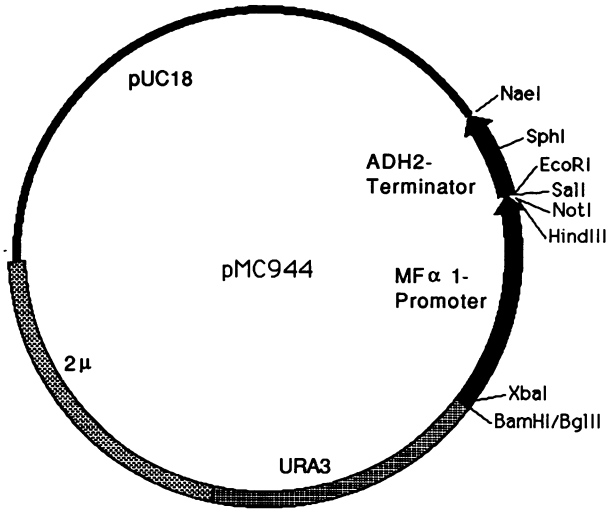
MATERIALS AND METHODS

Strains, media, and general cloning methods. The genotypes of yeast strains used in this study are given in Table 1. *S. cerevisiae* strains were grown in YPD or SC-URA (synthetic complete medium lacking uracil) medium; yeast media and standard methods for manipulating yeast cells are as described by Sherman et al. (84). Temperature-sensitive strains were routinely grown at 23°C. In the case of the cDNA library, yeast transformations were performed by the spheroplast method (36). Other transformations were carried out by the lithium acetate procedure (80). Yeast plasmid DNA was isolated by the method of Hoffman and Winston (37) and amplified by transformation into *Escherichia coli*. Yeast genomic DNA was prepared as described by Ausubel et al. (3). A qualitative glycogen test was performed by inverting plates over solid iodine in a glass container (16). Standard DNA manipulations were as described by Sambrook et al. (78). cDNAs were sequenced on both strands by dideoxy nucleotide chain termination methods (T7 sequencing kit; Pharmacia, Uppsala, Sweden). In some cases, sequence reactions were carried out with *Taq* polymerase and dye-labeled dideoxynucleotides (Applied Biosystems), and the analysis was performed with an ABI DNA sequencer (model 373A). The GCG software package (version 7.0; Genetics Computer Group, Inc., Madison, Wis.) was used for sequence analysis. The PIR (release 36.0) and SwissProt (release 20.0) data bases were searched with the FASTA algorithm of Pearson and Lipman (65). Polymerase chain reaction (PCR) incubations were for 20 cycles at 94°C for 40 s, 55°C for 45 s, and 72°C for 3 min in a DNA Thermal Cycler (Perkin-Elmer Cetus). The reaction conditions were as described by the manufacturer (Perkin-Elmer Cetus), with 50 pmol of each primer and 1 to 5 μg of template DNA. Yeast genomic DNA used as PCR template was prepared from wild-type strain S228C.

cDNA library construction. Total RNA was prepared from ovary oocytes by extraction with guanidinium thiocyanate at a low temperature (32). Prior to this extraction, oocytes were freed of ovarian tissues and follicle cells by treatment with collagenase (70). Poly(A)$^+$ RNA was isolated by chromatography on oligo(dT)-cellulose. cDNA was synthesized with the Amersham cDNA synthesis kit according to the manufacturer's recommendations, except for the following modifications. First-strand cDNA synthesis was carried out with a *Sal*I-oligo(dT) primer and Super RT reverse transcriptase (avian myeloblastosis virus reverse transcriptase; P.H. Stehelin & Cie AG, Basel, Switzerland). Second-strand cDNA was synthesized by RNase H-mediated replacement synthesis. *Eco*RI-*Not*I adaptors were ligated to the double-stranded cDNA. After digestion of the cDNA with *Eco*RI, excess adaptors as well as short cDNA products were removed by spin column chromatography (Sephacryl S-400; Pharmacia, Uppsala, Sweden). The cDNA was concentrated by ethanol precipitation and cloned into λgt11 arms precleaved with *Eco*RI (Promega, Madison, Wis.). Ligated λ DNA was packaged in vitro with the Gigapack plus packaging kit (Stratagene, La Jolla, Calif.) and amplified in *E. coli* Y1088. A library of 4.5×10^6 independent clones was obtained. λ DNA was prepared from plate lysates (100 plates) of the amplified library and purified by cesium chloride gradient centrifugation. One milligram of this λ DNA was digested with *Sal*I and *Not*I to recover the cDNA inserts, which were then subcloned into the yeast expression plasmid pMC944 (see below). Plasmid pMC944 was prepared for cDNA insertion by two consecutive *Sal*I and *Not*I digestions. The gel-purified and ethanol-precipitated cDNA inserts were ligated to pMC944 at a molar ratio of 1:1. A total of 1.7 μg of cDNA was ligated to 4.0 μg of vector. Transformation of *E. coli* DH5α resulted in a library of ~400,000 oriented clones. Colonies were scraped from the surface of the plates, and plasmids were recovered from the pooled bacteria by alkaline lysis followed by CsCl centrifugation. This DNA was used directly to complement *S. cerevisiae* mutants. The quality of the library was assessed by analysis of the plasmids recovered from 24 colonies selected at random.

The expression plasmid pMC944 (Fig. 1A) is based on plasmid YEp352 (35). To tailor YEp352 to our cloning needs, the multicloning site of this plasmid, which is derived from pUC18, was replaced by a synthetic polylinker that creates a different set of restriction sites (Fig. 1B). To convert the plasmid with the new polylinker into an expression plasmid, a 920-bp *Bgl*II-*Hin*dIII fragment carrying the promoter sequences of the *MFα1* gene (nucleotides −921 to −1 [see Fig. 9 in reference 24]) was subcloned between the *Bam*HI and *Hin*dIII sites, and a 460-bp *Eco*RI fragment carrying the *ADH2* terminator sequences (nucleotides 2246 to 2714 [Fig. 2 in reference 75]) was inserted between the *Eco*RI and *Nae*I sites. The *Hin*dIII restriction site at the 3′ end of the *MFα1* promoter was created by ligation of the 840-bp *Bgl*II-*Hin*fI fragment excised from a plasmid containing the entire *MFα1* gene with a pair of annealed synthetic oligonucleotides (81-mers) carrying the sequences spanning the *Hin*fI site to the end of the promoter and converting the starting ATG into a *Hin*dIII site. The *ADH2* terminator sequences were generated by PCR with a pair of primers containing the required restriction sites within their 5′ tails. The forward primer (5′-GGCGAATTCTAAGCGGATCTCCTATGCCTTCACG-3′) overlapped the stop codon (boldface) and carried an *Eco*RI site (underlined); the reverse primer (5′-GGCGGC CGGCCGCGTGAGGGTTCAATAATTGAAATTAT

A:



B:

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AGCTAGGATCCCAAAGCTTGGCGGCGGCCGCCGATCGTCGACTGGAATTCGCGGCCGGCCGCC*
*  TCCTAGGGTTTCGAACCGCCGCCGGCGGCTAGCAGCTGACCTTAAGCGCCGGCCGGCGGTTAA
    BamHI   HindIII      NotI        SalI      EcoRI      NaeI
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FIG. 1. The expression vector. (A) Schematic drawing of plasmid pMC944. The plasmid is derived from YEp352 (35). The yeast elements (filled boxes) include the 2μm origin of replication, the *URA3* gene, the *MFα1* promoter, and the *ADH2* terminator. The direction of transcription from the yeast promoter is indicated. The relevant restriction sites are shown. (B) The sequence of the multicloning site.

AGGGTGG-3′) carried a *Nae*I site (underlined). The accuracy of the construct was confirmed by sequencing.

Isolation of plasmids complementing the *cdc15* mutation. A total of 500 μg of the cDNA library was used to transform the yeast strain WS167-25 by the spheroplasting method (36). Transformants were selected on SC-URA plates at the permissive temperature (23°C). Transformants embedded in the top agar were scraped from the agar plates and resuspended in SC-URA. The optical density at 600 nm of the cell suspension was measured, and ~10^6 cells were plated onto SC-URA plates. These plates were incubated at 37°C for 1 week. Colonies that grew at the restrictive temperature were picked for further analysis.

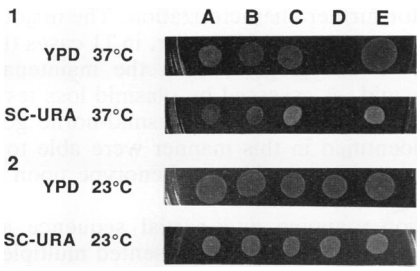

FIG. 2. Three *Xenopus* clones can suppress the *cdc15* mutation. The *cdc15* strain WS167-25 transformed with the indicated expression plasmids was spotted onto either YPD or SC-URA plates after 24 h of growth in selective medium at 23°C. Incubation was for 2 days at either 37°C (panel 1) or 23°C (panel 2). Plasmids: A, pF114; B, pF106; C, pF112; D, pMC944; E, pMC978.

Construction of a double mutant, *cdc15 ras2*. The *ras2*::*LEU2* disruption allele was constructed in vitro by insertion of the *LEU2* gene into the coding region of the *RAS2* gene (18). The construction started with the creation of two unique restriction sites flanking the coding region of the *RAS2* gene by PCR amplification. The forward primer (5′-GGGATCCGGGATCCTCATTCACTCCTTATCTGAC TCTTC-3′) contained two *Bam*HI sites (underlined) within its 5′ tail and the reverse primer (5′-AAAGCTTAAAGCT TAATAAGCAATATAAAAACAGTCAC-3′) contained two *Hin*dIII sites (underlined). The *Bam*HI-*Hin*dIII-digested PCR product was cloned into pUC18, and subsequently, the *LEU2* gene (excised as 4.2-kb *Pst*I fragment from the yeast vector YEp13 [11]) was subcloned into the unique *Pst*I site of the *RAS2* gene, located 65 amino acids from the N terminus. Replacement of the chromosomal *RAS2* gene by the *ras2*::*LEU2* allele was achieved by one-step gene disruption (73). The *ras2*::*LEU2* construction was excised from the pUC vector by digestion with *Bam*HI and *Hin*dIII, and the linearized fragment was used to transform strain WS167-25 to leucine prototrophy. Several Leu^+ transformants were screened for the disruption by phenotypic (elevated glycogen accumulation) and Southern blot analysis of genomic DNA. The double mutant strain was named WS183.

Plasmid constructions. (i) *RAS* overexpression plasmids. PCR amplification was used to create *Not*I and *Sal*I restriction sites at the 5′ and 3′ ends, respectively, of the *RAS1* and *RAS2* coding region (18). The following pairs of primers

were used: *RAS1*, 5′-GGCGGCCGCCCGGCGGCCGCATG CAGGGAAATAAATCAACTATAA-3′ and 5′-GGTCGAC CGGTCGACTCAACAAATTATACAACAACCACCAC-3′; *RAS2*, 5′-GGCGGCCGCCCGGCGGCCGCATGCCTTTGA ACAAGTCGAACATAA-3′ and 5′-GGTCGACCGGTCGA CTTAACTTATAATACAACAGCCACCCG-3′. Restriction sites are underlined; the start codon and stop anticodon are in boldface type. The *Not*I-*Sal*I-digested PCR products were subcloned into the *Not*I and *Sal*I sites of pMC944 to generate the expression plasmids pWS1108 (*RAS1* gene) and pWS1107 (*RAS2* gene).

(ii) *PDE2* expression plasmid. Plasmid p6a12 was constructed by subcloning a 3-kb *Bam*HI-*Bgl*II fragment containing the complete *PDE2* gene (79), isolated from plasmid pNAS4-1 (26) into the *Bam*HI site of YEp352 (35).

(iii) *MSI1* expression plasmid. To construct pWS889, a 2.2-kb *Hin*dIII-*Xho*I fragment encompassing the *MSI1* gene (74) was isolated from plasmid pNAS1-1 (26) and subcloned into the *Hin*dIII and *Sal*I sites of YEp352 (35).

(iv) *CDC15* expression plasmid. The *CDC15* gene, coding region, and 5′ and 3′ flanking sequences were provided by A. Amon on an ~4.3-kb *Bgl*II-*Sma*I fragment in a pUC vector. Following the addition of *Bgl*II linkers to the *Sma*I-digested *CDC15* plasmid, the gene was inserted as a *Bgl*II fragment into the *Bam*HI site of YCp*lac*33 (29) and YEp352 (35) to generate plasmids pMC978 and pMC977, respectively.

Cell cycle synchronization and cAMP determination. Synchronized cultures were obtained by pheromone treatment. Cells (*BAR*$^+$) were grown to an optical density at 600 nm of 0.1 (57) and arrested in G_1 by addition of α-factor at a final concentration of 10 μg/ml and incubation for 210 min at 23°C (99). Cells were then collected, washed with fresh medium, inoculated into a 0.5× volume of medium, to an optical density at 600 nm of ~0.4, and further incubated at 37°C. Samples were taken at 30-min intervals following removal of the pheromone for cAMP determination, cell counts, morphological examination, and budding index analysis.

To measure cAMP, cells were grown as noted in the figure legends. At the indicated times, aliquots were withdrawn from the cultures and cells were collected by filtration onto nitrocellulose filters. The filters were inverted into 35-mm petri dishes containing 1 ml of 1 N HCl and washed in this solution. The resuspended cells were transferred to prechilled microcentrifuge tubes containing glass beads, and cells were broken by vortexing three times in 1-min pulses. Tubes were chilled on ice for 1 min after each pulse. The homogenates were spun at 12,000 rpm for 5 min at 4°C, and the supernatants (cell extracts) were stored at −70°C. The cell extracts were thawed on ice, and 400-μl aliquots were either neutralized with an equal volume of 1 N NaOH prior to cAMP determination or lyophilized. In the latter case, the pellets were resuspended in Tris-EDTA buffer (50 mM Tris [pH 7.5], 4 mM EDTA). cAMP was measured with the Amersham cAMP [^{3}H] assay kit. Values were normalized to cell protein. Total protein in the cell extracts was determined as described by Bradford (9), with bovine serum albumin as the standard.

Northern (RNA) blot analysis. Total RNA was prepared from oocytes and embryos by the sodium dodecyl sulfate (SDS)-proteinase K method as previously described (69). RNA was resolved by formaldehyde-agarose gel electrophoresis and capillary blotted to Hybond C Extra membranes (Amersham). Radioactive antisense RNA probes were prepared by SP6 or T7 RNA polymerase transcription of linear plasmids (54). Probe hybridization in formamide solution and blot washing were performed by standard techniques (78).

Nucleotide sequence accession number. The EMBL accession numbers for *Xenopus* N-*ras* and β*TrCP* cDNA sequences are M97960 and M98268, respectively.

RESULTS

Construction of the expression library. A *Xenopus* oocyte cDNA library was constructed in the *S. cerevisiae* expression vector pMC944 (Fig. 1A). This high-copy-number plasmid gives good expression from the *MFα1* promoter and carries the *URA3* gene as a selectable marker. The vector does not contain an initiation codon and will therefore require that the ATG be encoded by the cDNAs in the library. The promoter and terminator sequences are separated by 25 nucleotides (Fig. 1B) containing *Not*I and *Sal*I restriction sites designed to subclone the cDNAs in an oriented manner and to facilitate transfer of a cloned cDNA from one vector into another without cutting within the insert. Two unique restriction sites, *Xba*I (position −894) in the promoter and *Sph*I (position 2560) in the terminator, allow the exchange of the complete expression cassette among different plasmid vectors.

Using mRNA prepared from total ovary oocytes and cDNAs synthesized in an oriented manner, an expression library of ~400,000 independent clones was obtained, with more than 90% of the clones containing cDNA inserts of an average size of 2 to 3 kb. The amount and coding capacity of mRNA in the oocyte has been measured and estimated to represent transcripts of 20,000 different structural genes with an average size of 2.2 kb (20). The quality of the library was further assessed by PCR. A series of specific primers designed to amplify several *Xenopus* genes corresponding to low (Vg1 [96])-, medium (B4, D7, enolase, and Eg1 [64, 82, 88, 89])-, and high (nucleoplasmin [14])-abundance maternal mRNAs were used in a standard PCR assay to test for the presence of the corresponding clones in the cDNA library. All but one of the genes, Vg1, were found to be present in the library. Vg1 is a rare message encoding a member of the transforming growth factor β family (96).

Two different *Xenopus* genes suppress the *CDC15* deficiency. DNA prepared from the cDNA expression library was used to transform the temperature-sensitive *cdc15* strain WS167-25. Yeast transformants were selected first for uracil prototrophy at the permissive temperature, 23°C. The primary transformants (~500,000) obtained were pooled, plated on medium lacking uracil, and incubated at the restrictive temperature, 37°C. Approximately 500 temperature-resistant colonies were obtained from ~10^6 plated cells, and 150 were retrieved for further characterization. The majority of these proved to be revertants. However, in 11 cases the ability to grow at 37°C cosegregated with the maintenance of the library plasmid, as assessed by plasmid loss tests, showing that the rescue was due to a plasmid-borne gene. The 11 plasmids identified in this manner were able to rescue the *cdc15* temperature sensitivity phenotype upon reintroduction into WS167-25 cells.

Restriction mapping and partial sequence analysis revealed that the 11 plasmids represented multiple isolates of three different cDNA clones that could be further grouped into two classes (Fig. 2). Plasmids pF106 (eight independent isolates) and pF112 (two independent isolates) represent one class and contain overlapping inserts, indicating that two different cDNAs derived from the same gene had been isolated. The difference resides in the 5′ end, pF112 being

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 -5                                                                AAGAA
  1   ATGACAGAATATAAACTGGTGGTGGTGGGAGCAGGAGGTGTTGGGAAAAGTGCCTTAACA
  1   M  T  E  Y  K  L  V  V  V  G  A  G  G  V  G  K  S  A  L  T
 61   ATCCAGCTTATACAGAACCATTTTGTAGATGAGTATGATCCTACTATCGAGGATTCCTAT
 21   I  Q  L  I  Q  N  H  F  V  D  E  Y  D  P  T  I  E  D  S  Y
121   AGAAAACAGGTAGTGATTGATGGAGAGACTTGCCTGTTGGATATTTTAGACACGGCTGGT
 41   R  K  Q  V  V  I  D  G  E  T  C  L  L  D  I  L  D  T  A  G
181   CAAGAGGAATACAGTGCTATGCGTGACCAGTATATGAGGACCGGGGAAGGATTTCTGTGT
 61   Q  E  E  Y  S  A  M  R  D  Q  Y  M  R  T  G  E  G  F  L  C
241   GTATTTGCTATTAATAACAGCAAGTCTTTTGCTGATATTAATGCCTACAGGGAACAGATA
 81   V  F  A  I  N  N  S  K  S  F  A  D  I  N  A  Y  R  E  Q  I
301   AAACGAGTGAAAGACTCAGATGATGTCCCCATGGTGCTTGTTGGAAATAAATGCGATTTG
101   K  R  V  K  D  S  D  D  V  P  M  V  L  V  G  N  K  C  D  L
301   CCAAGCAGAACCGTGGATACCAAGCAAGCCCAAGAGCTTGCCAGAAGCTATGGAATTCCT
121   P  S  R  T  V  D  T  K  Q  A  Q  E  L  A  R  S  Y  G  I  P
421   TTCATCGAGACCTCGGCAAAGACAAGACAGGGGGTAGAAGATGCATTTTACACCTTGGTT
141   F  I  E  T  S  A  K  T  R  Q  G  V  E  D  A  F  Y  T  L  V
481   AGAGAAATTCATCAGTATAGAATGAAGAAATTGGACAGCAGTGAAGATAACAATCAAGGA
161   R  E  I  H  Q  Y  R  M  K  K  L  D  S  S  E  D  N  N  Q  G
541   TGTATCCGAATTCCCTGCAAGCTTATGTAATCAGACCTTTGTTTTCCTATGGAAGAGTCC
181   C  I  R  I  P  C  K  L  M  *
601   CACTCTTTCCAAAAGCCATTCGCACAAGATACGACTGCTATCAATCCAAACAACACTTTT
661   CAATACCACTTACCTCCTTAACTCATGGTCGGATCACTGAACATGTACTTAAACCTGTGG
721   AAGAACTTGCCAAACTAGACGGCAAACTAGATGCTGATTATGTTTTTTTTTTGTTTTTGT
781   TTTTTTTTTTGGACAAGACACAGCCAACTGGAACTGTTTGTGGTAACCCAGGTAACATCA
841   ATCGGTGAGACAGAGCATTACCTGGCTATGCCCAATGTCTTGCTGTTATGCCCTCATCTC
901   ATGGTTTTAACAAATTGTTGAGCACCTCTATTTTTCATTTACCAAGGTTAGGAGGCAAAA
961   CATTCAAACCGTGTTCAATAACTTCTCTCAGGCCAATATGAAGGGCAATCTTTGACTTAA
1001  AAGACAACACTGTAAATGAAAAAAAAAAAAAAAAAAAAAAAAAAA
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FIG. 3. Nucleotide and predicted amino acid sequences of *Xenopus* N-*ras*. The stop codon is indicated with an asterisk.

the longer clone. Plasmid pF114 (one isolate) represents the other class and had a different sequence and restriction pattern. Initial experiments suggested that the cells transformed with this plasmid grew relatively poorly in SC-URA plates at the restrictive temperature; therefore, most of the experiments described in this report were conducted on YPD, though the proper SC-URA controls were always included. The suppressors did not rescue the *cdc15* mutation when expressed at low dosage on a *CEN* vector (data not shown). To establish that suppression is specific for *cdc15*, we examined the ability of the suppressors to rescue cell cycle mutations in two different complementation groups. The temperature sensitivity phenotypes of two yeast strains with a genetic background similar to the one of our *cdc15* strain, WS167-25, but carrying mutations in either *cdc28*, the key mitotic regulator (72, 90), or *cdc36*, a cell cycle gene of the mating pheromone response pathway (5), were not rescued by expression of plasmids pF106, pF112, and pF114 (data not shown).

pF114 encodes a *Xenopus* N-Ras protein. The sequence of the cDNA insert of plasmid pF114 is shown in Fig. 3. The cDNA has a length of 1,050 bp and encodes a polypeptide of 189 amino acids. The molecular mass of the predicted protein is 21 kDa. The amino acid sequence was compared with those in the protein data bases by using the FASTA program (65). The computer search identified this product as a member of the Ras protein family. The results of a multiple alignment analysis of *Xenopus* pF114 gene product; human N-, H- and K-Ras proteins (15, 85, 92); and *Xenopus* K-Ras (2) are shown in Fig. 4. The Ras proteins can be divided into three regions. The region corresponding to the catalytic domain, amino acids 1 to 165, is highly conserved, with the amino-terminal 86 amino acids being identical in these five proteins. The next 20 amino acids are known to be divergent among the three members of the Ras family, although within each type (H, K, or N) the amino acid sequence of this heterogeneous region is relatively well conserved. The last four residues correspond to the CAAX box, a motif subjected to posttranslational modifications. pF114 amino acid sequence shows highest similarity to the human N-Ras protein: 93% identity over the entire protein sequence. In the heterogeneous region the protein encoded by pF114 is 65% identical to human N-Ras but only 5% identical to *Xenopus* K-Ras, whereas the heterogeneous regions of *Xenopus* K-Ras and human K-Ras are also 65% identical. These results suggest that pF114 encodes a *ras* cDNA of the N-*ras* type. The insert of pF114 will hereafter be referred to as *Xenopus laevis* N-*ras*.

Overexpression of the yeast genes *RAS1* and *RAS2* does not suppress the *cdc15* mutation. The structural, biochemical, and functional homology between yeast and mammalian Ras proteins is well documented (4, 93). The essential function of the two yeast Ras proteins is to activate adenylate cyclase (94), and although this function may not have been conserved in other species, human *ras* genes can substitute for the loss of both yeast *RAS* genes and modulate yeast adenylate cyclase (12, 21, 39). Thus, the finding that *Xenopus* N-*ras* was able to suppress the *cdc15* mutation prompted us to examine whether the yeast *RAS* genes would also suppress this mutation. To answer this question, we over-

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XN-ras  MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINN  86
hN-ras  --------------------------------------------------------------------------------  86
hH-ras  --------------------------------------------------------------------------------  86
hK-ras  --------------------------------------------------------------------------------  86
XK-ras  --------------------------------------------------------------------------------  86

XN-ras  SKSFADINAYREQIKRVKDSDDVPMVLVGNKCDLPSRTVDTKQAQELARSYGIPFIETSAKTRQGVEDAFYTLVREIHQ       165
hN-ras  --------L-----------------------------------H---K----------------------------R-       165
hH-ras  T---E--HQ-----K------------------AA---ESR------------Y----------------------R-       165
hK-ras  T---E--HH-----K-----------------------------D-------------------D----------RK       165
XK-ras  T---E--HH---- N---------------------------------K----------------------------RK       165

XN-ras  YRMKKLDSSEDNNQGCIRIPCKLM  189
hN-ras  ------N--D-GT---MGL--VV-  189
hH-ras  HKLR--NPPDESGP--MSCK-V-S  189
hK-ras  HKE-MSKDGKKKKKKSKSTK-VI-  189
XK-ras  HKE-ISN.GKKKKS.SK.RK-VVL  189
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FIG. 4. Comparison of *Xenopus* N-Ras amino acid sequence with the corresponding sequences of other members of the *ras* gene family. The dashes indicate amino acids identical to *Xenopus* N-Ras; dots indicate amino acid gaps introduced to generate an optimal alignment. The CAAX box is in boldface type. The sequences for human N-Ras (92), H-Ras (15), K-Ras (85), and *Xenopus* K-Ras (2) were retrieved from the data bases.

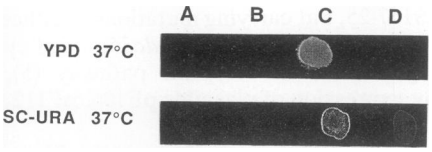

FIG. 5. The *cdc15* mutation is not suppressed by overexpression of the two yeast *RAS* genes. The *cdc15* strain WS167-25 transformed with the indicated plasmids was spotted onto either YPD or SC-URA plates after overnight growth in selective medium at 23°C. Incubation was for 3 days at 37°C. Plasmids: A, pWS1107 overexpressing *S. cerevisiae RAS2* gene; B, pWS1108 overexpressing *S. cerevisiae RAS1* gene; C, pF114 overexpressing *Xenopus* N-*ras* gene; D, pMC944, control plasmid.

expressed *S. cerevisiae RAS1* and *RAS2* genes in a *cdc15* background. The expression plasmids pWS1107 and pWS1108 were constructed by cloning the *RAS1* and *RAS2* genes, respectively, in the library plasmid pMC944 (for details, see Materials and Methods). To verify overexpression from the two *RAS* constructs, we transformed a wild-type strain (WS165-2B) with either the *RAS1* or the *RAS2* expression plasmids and scored glycogen accumulation. A typical phenotype of *RAS*-overexpressing yeast cells is their failure to accumulate storage carbohydrate when entering into stationary phase (94). The *RAS1* and *RAS2* transformants showed decreased levels of glycogen accumulation (data not shown), thus confirming that the constructions produced functional Ras proteins. Subsequently, the *cdc15* strain WS167-25 was transformed with the two yeast *RAS* plasmids, pWS1107 and pWS1108; the *Xenopus* N-*ras* plasmid, pF114; and the library plasmid, pMC944, and individual transformants were tested for their abilities to grow at the restrictive temperature (37°C) in synthetic (SC-URA) as well as in rich (YPD) media. Figure 5 shows that, whereas *Xenopus* N-*ras* can suppress the *cdc15* defect, neither yeast gene is able to do so. Clearly N-*ras*-transformed *cdc15* cells were able to grow at the restrictive temperature, although they grew considerably better in rich media than in synthetic media, as mentioned above. In contrast, no growth of cells transformed with yeast *RAS* was observed at the restrictive temperature, and cells were arrested with a typical *cdc15* morphology. Since activation of the yeast RAS-cAMP pathway in an unregulated fashion may lead to rapid loss of viability (94), we also compared the growth rates of these transformants at the permissive temperature. In this case, all transformants grew equally well (data not shown), indicating that overexpression of various Ras proteins does not lead to significantly decreased viability. Furthermore, a plasmid loss test showed that the *RAS* overexpression plasmids were lost at about the same rate as the vector plasmids. These results imply that there is a fundamental difference in the functions of yeast Ras and *Xenopus* N-Ras proteins.

***X. laevis* N-Ras decreases cAMP levels in *S. cerevisiae*.** Next we examined whether the *Xenopus* N-Ras protein complements the *cdc15* mutation via the RAS-cAMP pathway or by an unknown mechanism. Because yeast *RAS* controls adenylate cyclase activity, involvement of the RAS-cAMP pathway in a biological process invariably leads to changes in intracellular cAMP levels. These changes in turn can be followed by terminal phenotypes of other functions controlled by cAMP-dependent protein kinase, such as the commitment of the cell to store or to break down storage carbohydrates. High levels of cAMP reduce glycogen levels, whereas low levels of cAMP promote glycogen storage (53).

TABLE 2. Ability of overexpressed *RAS* genes to suppress the temperature sensitivity defect of the *cdc15* mutation and their effect on glycogen storage[a]

Transforming plasmid	Growth		Glycogen storage	
	23°C	37°C	23°C	37°C
pMC944	+++	−	+	+
pF114	+++	+++	++	++
pWS1108	+++	−	−	−
pWS1107	+++	−	−	−

[a] Strain WS167-25 (*cdc15*) was transformed with pMC944-based plasmids overexpressing *Xenopus* N-*ras* (pF114), *S. cerevisiae RAS1* (pWS1108) and *S. cerevisiae RAS2* (pWS1107), and with the vector plasmid alone (pMC944). Cells were cultured as described in the legend to Fig. 5. Growth was scored after 3 days of incubation at 23 and 37°C in YPD plates. The plates were then stained by iodine vapors. All experiments were repeated at least three times.

Therefore, involvement of the RAS-cAMP pathway can be easily monitored by a color assay based on iodine staining of glycogen. To investigate whether *Xenopus* N-Ras interacts with the yeast RAS-cAMP pathway, the four yeast transformants described in the previous section were assayed for glycogen accumulation. *cdc15* cells expressing *Xenopus* N-*ras* accumulated more glycogen than *cdc15* cells transformed with the control plasmid (pMC944). In contrast, *cdc15* cells transformed with the two yeast *RAS* genes (pWS1107 and pWS1108) had reduced glycogen levels (Table 2). Similar results were obtained when a wild-type strain, WS165-2B, was transformed with the same set of plasmids and glycogen accumulation was measured (data not shown). These data suggest that in contrast to yeast Ras, *Xenopus* N-Ras turns off the RAS-cAMP pathway. Expression of the other suppressor gene, β*TrCP* (see below), in the *cdc15* strain did not influence the RAS-cAMP pathway.

Having shown that expression of *Xenopus* N-*ras* can complement the growth defect of a *cdc15* mutant strain and that this expression leads to higher glycogen content due, presumably, to a lowered cAMP level, we measured cAMP concentration in *cdc15* cells transformed with either the *Xenopus* N-*ras* plasmid or the pMC944 vector. cAMP measurements were performed on dividing cells grown to stationary phase at the permissive temperature and on cells blocked by the *cdc15* mutation. Duplicate cultures inoculated under the same conditions and grown at 23°C for 24 h were split into two; one half was kept at the permissive temperature, 23°C, while the other half was shifted to 37°C. Both cultures were incubated for another 3 h, and their cAMP levels assayed at this point. Figure 6A shows that the levels of cAMP in cycling cells (23°C) are about twofold lower in *Xenopus* N-*ras*-transformed cells than in vector-transformed *cdc15* cells. The difference in cAMP levels at the permissive temperature is in agreement with the data for glycogen accumulation (Table 2). Figure 6B shows that *cdc15* cells expressing the *Xenopus* N-*ras* gene have lower levels of cAMP than their control counterparts when arrested at the restrictive temperature.

Decreasing intracellular cAMP concentration is sufficient to complement the *cdc15* mutation. Next, we determined whether lowering of cAMP levels alone is sufficient to complement the *cdc15* mutation. To this end, we constitutively lowered intracellular cAMP levels in a *cdc15* mutant strain by three independent methods and analyzed the phenotype of those cells. First, we constructed a *cdc15 ras2* double mutant. This was done by substitution of the wild-type *RAS2* gene in the *cdc15* strain WS167-25 with a

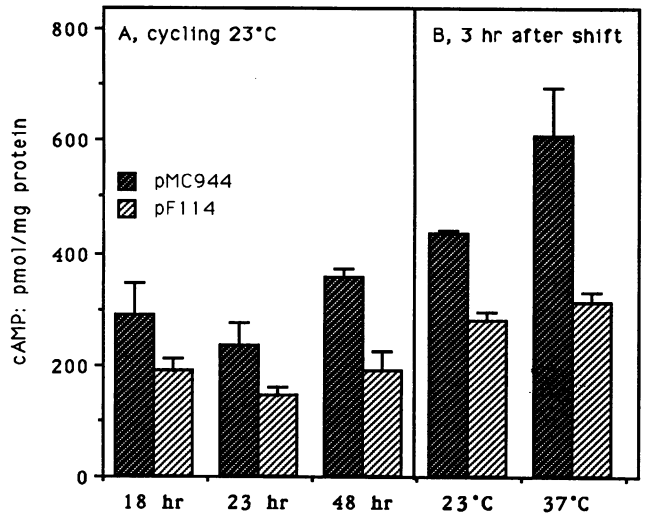

FIG. 6. Effects of *Xenopus* N-*ras* on the cAMP levels of *cdc15* cells. Cells from cultures that have reached stationary phase on synthetic media at 23°C were used to inoculate fresh medium (YPD) and incubated at the indicated temperatures. Fractions (10 ml) of the cultures were harvested at the indicated times and assayed for cAMP content and total cell protein. (A) Dividing cells grown to stationary phase. Cells were grown at 23°C. (B) Dividing versus *cdc15*-arrested cells. Cultures growing at 23°C were divided into two: one half was kept at 23°C (permissive temperature), while the other half was shifted to 37°C (restrictive temperature). Both cultures were incubated for an additional 3 h and harvested. The *cdc15* strain was transformed with the control plasmid (pMC944) or with pF114. Duplicate samples were taken for each time point, and each sample was assayed twice. Error bars represent standard deviations.

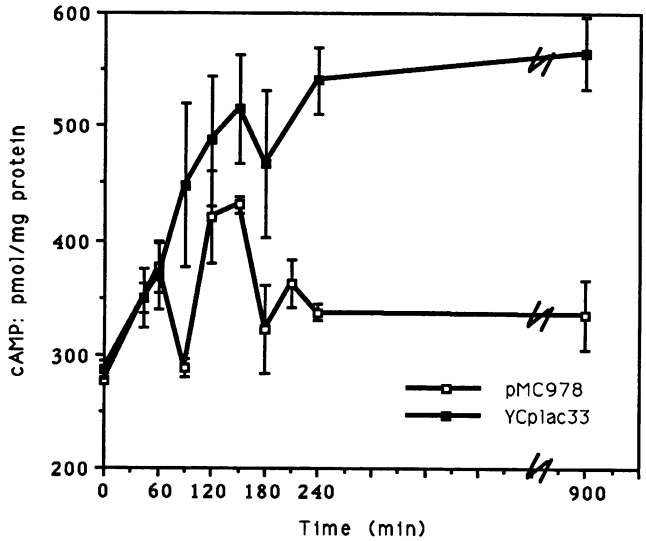

FIG. 8. Intracellular cAMP levels in *cdc15* strains during a synchronous cell cycle. Cells were synchronized in G_1 by treatment with α-factor (10 μg/ml) for 210 min at 23°C followed by release into medium without α-factor (at 0 min) and further incubation at 37°C. Samples (25 ml) were generally taken at 30-min intervals and assayed for cAMP content and total cell protein. Strain K838 (*cdc15*) was transformed with either plasmid pMC978 carrying the *CDC15* gene or the yeast vector YCp*lac*33. The graph represents averages of two experiments. Duplicate samples were taken for each time point, and each sample was assayed twice. Error bars represent standard deviations.

ras2::*LEU2* disruption allele. Toda et al. (94) have shown that *ras2* mutants have fourfold-lower levels of cAMP than wild-type cells. In a *cdc15* background, loss of *RAS2* function also leads to lower cAMP levels, as estimated by the corresponding increase in glycogen accumulation (data not shown). Reduced cAMP content was sufficient to overcome the *cdc15* block, since *cdc15 ras2* cells were able to grow at the restrictive temperature of the *cdc15* mutation (Fig. 7, panel 1). To further confirm these results, we overexpressed in a *cdc15* strain two yeast genes involved in the control of intracellular cAMP levels: *PDE2* and *MSI1*. *PDE2* encodes a high-affinity cAMP phosphodiesterase that catalyzes the hydrolysis of cyclic 3′,5′-nucleoside monophosphates to the corresponding 5′-nucleoside monophosphates (79). *MSI1* is a downregulator of RAS-cAMP pathway with unknown function (74). The high-copy-number plasmids pWS889 and p6a12 carrying the yeast *MSI1* and *PDE2* genes, respectively, were used to transform the *cdc15* strain WS167-25. Transformants were allowed to grow at the permissive temperature and were then shifted to the restrictive temperature. Growth was clearly observed in *cdc15* cells overexpressing *PDE2* or *MSI1*, while *cdc15* cells transformed with the vector were nonviable (Fig. 7, panel 2).

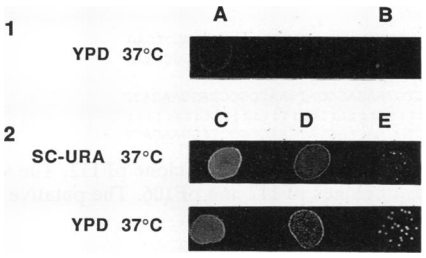

FIG. 7. Suppression of the *cdc15* mutation by lowering intracellular cAMP. (Panel 1) The *cdc15 ras2* double mutant suppresses the lethality of the *cdc15* mutation. Yeast cells were spotted onto a YPD plate after overnight growth in YPD medium at 23°C. Incubation was for 2 days at 37°C. Strains: A, WS183 (*cdc15 ras2*); B, WS167-25 (*cdc15*). (Panel 2) Overexpression of genes that negatively regulate the RAS-cAMP pathway. The *cdc15* strain WS167-25 transformed with the indicated plasmids was spotted onto either YPD or SC-URA plates after overnight growth in selective medium at 23°C. Incubation was for 3 days at 37°C. Plasmids: C, p6a12 carrying the *PDE2* gene; D, pWS889 carrying the *MSI1* gene; E, YEp352, control plasmid.

Intracellular levels of cAMP during the cell cycle. To evaluate whether the *CDC15* gene product is required to reduce cAMP during mitosis, we measured intracellular cAMP levels during a synchronous cell cycle. To mimic wild-type conditions, a *cdc15* strain with an **a** mating type, K838, was transformed with plasmid pMC978, a *CEN* plasmid carrying the *CDC15* gene under the control of its own promoter, while the same strain transformed with the yeast *CEN* vector YCp*lac*33 (29) was used as the *cdc15* control. Synchronized cultures were obtained by releasing cells from alpha-factor-induced G_1 arrest. The results of this analysis are shown in Fig. 8. Expression of the *CDC15* gene in a *cdc15* background yields a transient decrease in cAMP at around the time of the *cdc15* arrest (90 min). Although the magnitude of this fluctuation is not dramatic, it is reproducible. A similar effect was observed when the *CDC15* gene was expressed in a high-copy-number plasmid (pMC977

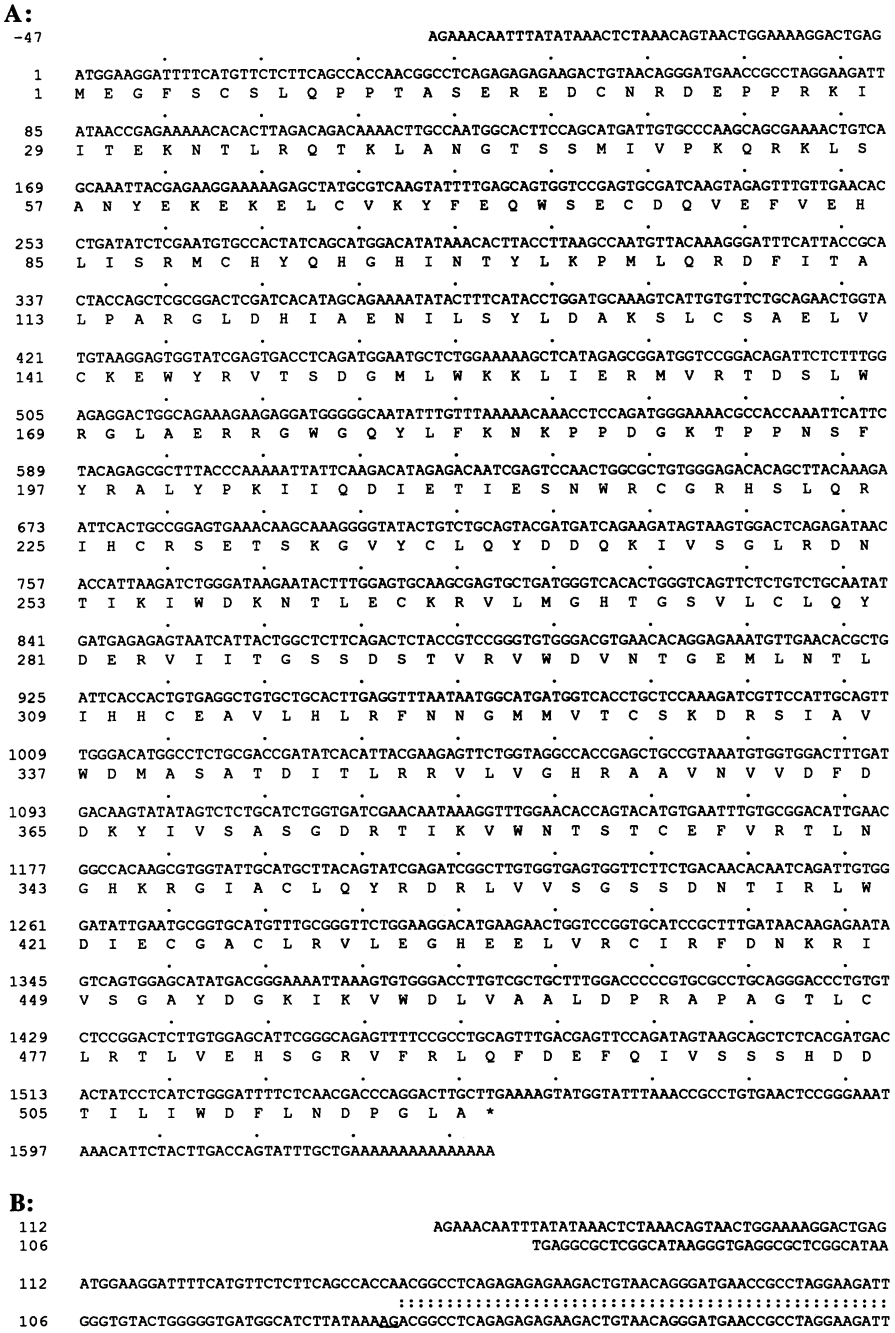

FIG. 9. Nucleotide and predicted amino acid sequences of the β*TrCP* gene. (A) The sequence encoded by clone pF112. The stop codon is indicated with an asterisk. (B) Comparison of the nucleotide sequence at the 5′ ends of clones pF112 and pF106. The putative AG splice acceptor site is underlined. The identity of the residues 3′ of the proposed splicing site is indicated by colons.

[data not shown]). In contrast, no decrease in cAMP levels was observed in the *cdc15* cells (vector transformed), in which cAMP levels remained higher after the *cdc15* arrest. These results are in good agreement with previous data reported by Smith et al. (87) showing that cAMP levels fluctuate during mitosis and are lowest prior to and just after cell separation. The results are also in agreement with the data presented in Fig. 6. Taken together, these experiments demonstrate that *cdc15*-arrested cells have elevated cAMP levels.

Clone pF112 encodes a 60-kDa protein containing seven β-transducin repeats in its C-terminal domain. The cDNA insert of plasmid pF112 was sequenced completely, and the results are shown in Fig. 9A. The 1,686-bp sequence contained a single open reading frame of 518 amino acids, followed by 57 bp of noncoding sequence. The estimated molecular mass of the predicted protein product is 59.5 kDa. Upstream of the open reading frame there are 47 bp of noncoding region containing stop codons in all three reading frames. The C-terminal domain of the protein, amino acid

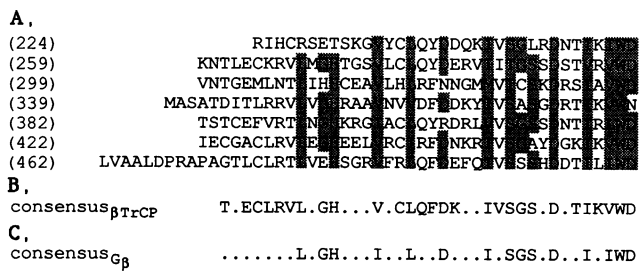

FIG. 10. (A) Alignment of the seven repeats of *Xenopus* β*Trcp*. Residues homologous to the key residues of the structural repeats of the β subunits of G proteins are shaded. The numbers in parentheses refer to amino acid residues based on the numbering used in Fig. 9A. The following amino acids are considered similar: A, L, V, I and M; D and E; K and R; N and Q; F and Y; and S and T. (B) Consensus sequence for a repeat unit derived from the seven repeats in the *Xenopus* βTrcp protein. The residues included are at or above a frequency of 0.40. (C) Previously reported consensus sequence for a unit repeat as derived from the repeats in five proteins of the β-transducin family (19).

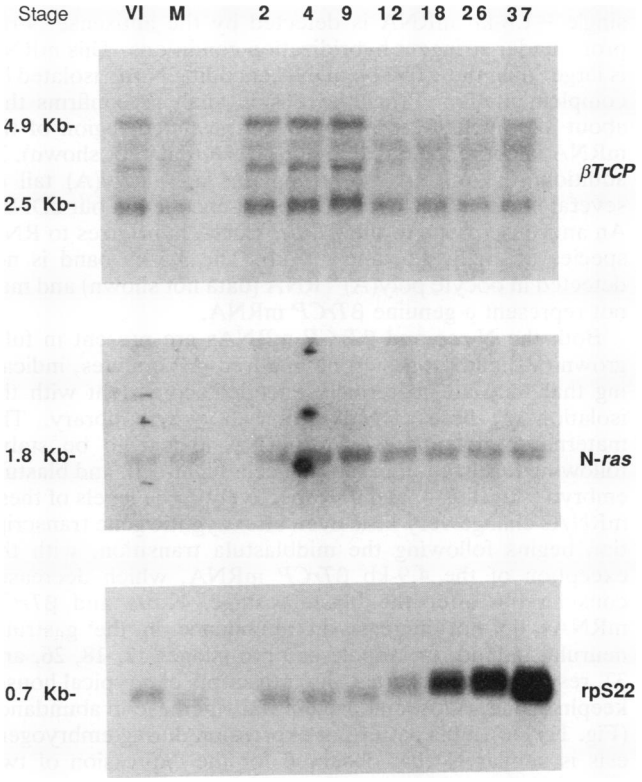

FIG. 11. Northern analysis of expression of β*TrCP* and N-*ras* mRNA during *Xenopus* development. Shown is the hybridization to two oocyte or embryo equivalents of total RNA per lane. Stages: VI and M, fully grown and progesterone-matured oocytes, respectively; 2, two-cell embryo; 4, eight-cell embryo; 9, blastula embryo; 12, gastrula embryo; 18, neurula embryo; 26, tailbud embryo; 37, a young tadpole, as described by Nieuwkoop and Faber (60). The bottom panel shows the hybridization of a control probe encoding *Xenopus* ribosomal protein S22 (rpS22) to the same blot used for N-*ras* hybridization. The rRNA content, which does not change appreciably during this period of development, was approximately equivalent in all lanes (not shown).

residues 214 to 518, shows a structural motif that is repeated seven times (Fig. 10A) and that was originally identified in the β subunit of transducin (25), a G protein involved in phototransduction. The β subunits of transducin and of other G proteins have a variable number of contiguous imperfect 43- to 68-amino-acid repeats characterized by the conservation of certain residues and their spatial relationships (25). The motif is now called the β-transducin repeat. A more detailed examination of the repeat domain revealed that the degree of homology to β-transducin, 24% identity and 50% similarity, extends over the entire length of the transducin molecule (340 amino acids) and is statistically significant. In the repeat domain the protein is also homologous (18 to 28% identity and 48 to 58% similarity) to the yeast members of the β-transducin protein family: Cdc4, Cdc20, Mak11, Msi1, Prp4, Ste4, and Tup1 (38, 66, 74, 83, 97, 98, 101) and to *Drosophila Enhancer of split* [*E*(*spl*), (33)]. A consensus sequence for the repeats in clone pF112 is shown in Fig. 10B. As reference, a consensus sequence derived from 25 versions of the repeat in five members of the β-transducin family (19) is shown in Fig. 10C. The N-terminal domain of the predicted protein, however, revealed no discernible homology to any known gene product, including the N-terminal domains of known G protein subunits. The new gene was named β*TrCP*, an acronym for β-transducin repeat-containing protein.

Sequence analysis of the 5′ end of clone pF106 and comparison to that of clone pF112 revealed sequence identity downstream of nucleotide 33 (Fig. 9B). Sequence analysis at this site indicates that clone pF106 might represent an unspliced version of pF112, since a canonical AG splice acceptor typical of intron-exon junctions is present at this position. We presume that an amino-terminally truncated protein is synthesized from clone pF106 with the methionine codon at position 139 as the starting ATG (Fig. 9A). This truncated protein is still able to complement the *cdc15* mutation, though slightly less efficiently than the full-length protein (Fig. 2).

β*TrCP* does not encode the *Xenopus* homolog of *CDC20*. The yeast gene *CDC20* encodes a protein of the β-transducin family (83). This protein has two domains and is very similar in length (519 amino acids) to the predicted product of *Xenopus* β*TrCP*. Although the overall sequence homology between the two proteins is poor, the N-terminal domains share 19.7% identical residues over a stretch of 150 amino acids and the C-terminal domains share the β-transducin repeats. Furthermore, overexpression of *CDC20* suppresses the temperature sensitivity defect of the *cdc15* mutation (1). To determine whether β*TrCP* is a functional homolog of the yeast *CDC20* gene, we expressed *Xenopus* β*TrCP* in *cdc20* cells and monitored whether the *Xenopus* gene product complements the loss of endogenous *CDC20* function and restores normal cell cycle progression. Transformation of a *S. cerevisiae cdc20* strain, K2773, with plasmids pF106, pF112, and pMC944 and subsequent incubation of the transformants at the restrictive temperature, 37°C, revealed no growth difference between vector transformants and transformants expressing β*TrCP* (data not shown). These results suggest that the *Xenopus* βTrcp protein cannot provide all the necessary functions performed by the Cdc20 protein. Despite their similar sequence motifs, *CDC20* and β*TrCP* are not functionally interchangeable.

Expression of N-*ras* and β*TrCP* during *Xenopus* development. We analyzed the steady-state mRNA levels for N-*ras* and β*TrCP* during early *Xenopus* development by Northern blot of total RNA from oocytes and embryos (Fig. 11). A

single ~1.8-kb mRNA is detected by the antisense N-*ras* probe under stringent hybridization conditions. This mRNA is larger than the 1,050-bp cDNA encoding N-*ras* isolated by complementation. Primer extension analysis confirms that about 420 nucleotides of the 5′ untranslated region of the mRNA is not represented in our cDNA (data not shown). In addition, the mRNA likely contains a 3′ poly(A) tail of several hundred nucleotides also not encoded in our cDNA. An antisense probe to the β*TrCP* cDNA hybridizes to RNA species of ≈2.5, 3.5, and 4.9 kb. The 3.5-kb band is not detected in oocyte poly(A)$^+$ RNA (data not shown) and may not represent a genuine β*TrCP* mRNA.

Both the N-*ras* and β*TrCP* mRNAs are present in fully grown (VI) and progesterone-matured (M) oocytes, indicating that they are maternally encoded, consistent with the isolation of these cDNAs from an oocyte library. The maternal N-*ras* and β*TrCP* mRNA appear to be stable following fertilization in the two-cell, eight-cell, and blastula embryo (stages 2, 4, and 9, respectively). The levels of these mRNAs change very little even after zygotic gene transcription begins following the midblastula transition, with the exception of the 4.9-kb β*TrCP* mRNA, which decreases considerably after the blastula stage. N-*ras* and β*TrCP* mRNAs do not increase in abundance in the gastrula, neurula, tailbud, or tadpole embryo (stages 12, 18, 26, and 37, respectively), whereas the transcripts of a typical housekeeping gene, ribosomal protein S22, increase in abundance (Fig. 11) (40). This pattern of expression during embryogenesis is similar to that observed for the expression of two cloned *Xenopus* K-*ras* genes (2, 6).

DISCUSSION

The *S. cerevisiae CDC15* gene product is required for the completion of the cell cycle (17). Cells which lack this function are blocked in mitosis with a high level of cdc2/Cdc28 kinase activity (55). The *CDC15* gene has been recently cloned and proposed to be a protein kinase (81). By complementation of the *S. cerevisiae cdc15* mutation with a *Xenopus* oocyte expression library, we have isolated two cDNAs that in high copy number are able to suppress the lethality of the mutation. One of these cDNAs encodes N-*ras*, and the other encodes a member of the β-transducin protein family, named β*TrCP*. A second screening of the pooled transformants resulted in the repeated isolation of the same two cDNAs (N-*ras* 4 times and β*TrCP* 110 times) plus three additional cDNAs that so far have been only partially characterized.

Functional differences among the Ras proteins. One of the cDNA clones isolated in this screen encoded *Xenopus* N-*ras*. Extensive genetic and biochemical analysis suggests that yeast Ras proteins are involved in the cAMP effector pathway. Indeed, yeast Ras proteins control the activity of adenylate cyclase, the enzyme that converts ATP into cAMP, and cAMP in turn activates the cAMP-dependent protein kinase (PKA). This kinase triggers a protein phosphorylation cascade responsible for a variety of cellular activities (reviewed in references 10, 28, and 53). In contrast, mammalian Ras proteins appear to signal to an unknown downstream effector that functions independently of cAMP and does not regulate the activity of adenylate cyclase (7, 8). Despite these differences, mammalian Ras proteins expressed in *S. cerevisiae* can supply essential RAS functions, stimulate adenylate cyclase (12, 21, 39) and interact with the *CDC25* gene product (68). The identification of a human homolog of *CDC25* has recently been reported (31). Moreover, two *S. cerevisiae* genes, *IRA1* and *IRA2*, that are negative regulators of *RAS*, encode functional homologs of mammalian *GAP* (91), the GTPase-activating protein. Thus, conservation in this transduction pathway seems to go beyond the structural similarities of the Ras proteins.

While many studies have contributed to establish the functional homology of the Ras proteins, only few have reported differences (13, 52). We find that overexpression of *Xenopus* N-*ras*, in contrast to that of the two yeast *RAS* genes, rescues the *cdc15* mutation. Expression of *Xenopus* N-Ras in *S. cerevisiae* reduced intracellular cAMP concentration, the opposite effect to that observed upon yeast RAS overexpression. Furthermore, we show that lowering intracellular cAMP levels is sufficient to suppress the *cdc15* mutation (see below). This explains the inability of *RAS1* and *RAS2*, which stimulate adenylate cyclase activity, to rescue the *cdc15* mutation. Since intracellular cAMP concentration is a function of both the synthesis and the degradation of the molecule, *Xenopus* N-Ras could cause a decrease in cAMP levels by either inhibition of adenylate cyclase or stimulation of phospodiesterase activity. It is possible, for example, that *Xenopus* N-Ras and yeast adenylate cyclase interact in a nonproductive manner, thereby either impairing the activity of the enzyme directly or sterically inhibiting interaction with endogenous Ras molecules.

Decreased intracellular cAMP levels are also required for *Xenopus* cell cycle progression. Meiotic maturation of *Xenopus* oocytes is modulated by cAMP concentration. Progesterone, the physiological inducer of maturation, lowers cAMP levels, whereas increased cAMP levels inhibit maturation (50, 51). Microinjections into *Xenopus* oocytes of human H-*ras*, H-*ras*$^{Val\text{-}12}$, and N-*ras*$^{Val\text{-}12}$ all elicit meiotic maturation (8, 77, 95). Though initially no measurable effect on cAMP levels was detected (8), recently H-*ras*-induced maturation has been shown to involve transient stimulation of phosphodiesterase activity, consistent with at least a temporary decrease in intracellular cAMP levels (76, 77). Thus, the same mammalian H-Ras which activates yeast adenylate cyclase to cause an increase in intracellular cAMP (12, 21) can, on the other hand, stimulate *Xenopus* phosphodiesterase to cause a decrease in cAMP levels (76, 77). Perhaps, then, the effect elicited by introduction of exogenous Ras into a cell is dictated by its ability or inability to productively interact with the endogenous Ras-mediated transduction pathway(s) rather than by its function in the cells from which it was derived.

The two kinases and the completion of mitosis. Our results suggest that a decrease in intracellular cAMP concentration is associated with suppression of the temperature-sensitive phenotype of the *cdc15* mutation. Reduction of cAMP in a temperature-sensitive *cdc15* strain by four different approaches, expression of *Xenopus* N-*ras*, disruption of the *RAS2* gene, overexpression of the high-affinity cAMP-phosphodiesterase (*PDE2* [79]), and overexpression of a negative regulator of the RAS-cAMP pathway (*MSI1* [74]), allows cells with a defective Cdc15 function to complete the mitotic cycle. A *cdc15* null allele, however, could not be rescued by this mechanism (data not shown), indicating that the presence of the defective *CDC15* gene product is required for the suppression. Since the cellular role of cAMP in yeast cells is to modulate the activity of the cAMP-dependent protein kinase (53), these results suggest that a key step of the cell cycle is dependent upon a phosphorylation event catalyzed by PKA. The following possibilities can be envisioned for a relationship between PKA and Cdc15. First, the Cdc15

kinase might directly or indirectly inhibit PKA. If so, the functions encoded by N-*ras*, *PDE2*, *MSI1*, and other genes that are able to downregulate PKA could compensate for the absence of a proper Cdc15 function. A second possibility is that phosphorylation by PKA inactivates Cdc15. In this case, overexpression of genes that inhibit PKA activity may help to stabilize the function of the mutant *cdc15* gene product. Alternatively, PKA might repress expression of the *CDC15* gene, in which case lower cAMP levels would result in synthesis of more Cdc15 protein. Lastly, Cdc15 and PKA could act upon a common substrate, as activator and inhibitor, respectively. In such a case, lack of activation by Cdc15 could be neutralized by inhibition of the inhibitor, PKA. The elucidation of a functional relationship or a possible molecular interaction between PKA and Cdc15 awaits further investigation.

Inactivation of the cdc2/Cdc28 kinase is essential for a cell to exit mitosis and both cyclin degradation and kinase dephosphorylation seem to be crucial events (49, 62). Mutant cyclins truncated at their N-terminal domains are resistant to degradation, causing the permanent activation of the cdc2/Cdc28 kinase and blocking dividing cells in mitosis (27, 56). *cdc15* mutants are arrested in a late stage of anaphase with a high cdc2/Cdc28 kinase activity (55), segregated chromosomes, and a fully assembled mitotic spindle (17). Because *CDC15* encodes a protein kinase (81) required for the completion of the cell cycle, it is tempting to speculate that the *CDC15* gene product, directly or through PKA, might be involved in the events associated with the destruction of cyclin and/or the concomitant inactivation of the cdc2/Cdc28 kinase. Events such as the phosphorylation of cyclin, the activation of the ubiquitin-conjugating enzymes, or of some other enzymatic steps leading to cyclin proteolysis and the subsequent dephosphorylation of the free cdc2/Cdc28 kinase are likely to be modulated by a balance of phosphorylations and dephosphorylations triggered by more than one kinase (30).

Homology between βTrcp and the β subunits of G proteins. The analysis of the amino acid sequence encoded by the *X. laevis* β*TrCP* gene suggests that the protein has two domains: an N-terminal domain without significant homology to any known gene product and a C-terminal domain composed of seven repeats which shares homology with the β subunits of G proteins. A consensus sequence for the repeats matches perfectly the consensus previously reported for the repeat motif of some members of the β-transducin family (19). On this basis, we tentatively named the *Xenopus* gene β*TrCP* (β-transducin repeat-containing protein).

Proteins in the β-transducin family fall into two classes: one class in which the proteins are composed almost entirely of the repeat domain, and a second class in which the repeats are restricted to the C-terminal domain. Proteins in the first group are known to serve as β subunits in the heterotrimeric G protein complexes that transduce receptor-generated signals (58). Mammalian β-transducins and *S. cerevisiae* Ste4 fall into this class (86, 97). Proteins in the second group are involved in activities as diverse as microtubule-dependent processes (Cdc20), catabolite repression (Tup1), regulation of the RAS-cAMP pathway (Msi1), RNA splicing (Prp4), DNA replication (Cdc4), and neurogenesis [E(spl)] (33, 66, 74, 83, 98, 101). There is no evidence that proteins of this group form part of trimeric G protein complexes. The repeat is nevertheless expected to be involved in protein-protein interactions. The finding that Tup1, a β-transducin repeat protein, and Ssn6, a tetratricopeptide repeat protein, are associated in a functional complex (41) has lent substantial support to this hypothesis. The presence of β-transducing repeats in *Xenopus* β*Trcp* suggests that it may interact with and/or regulate other proteins. βTrcp is the second protein of this type to be isolated from higher eukaryotes, the other one being *Drosophila* E(spl) (33). *CDC20* is a gene encoding a protein of the same family as β*TrCP* which also suppresses the *cdc15* mutation (1). However, *Xenopus* β*TrCP* and *S. cerevisiae CDC20* were found not to be functionally interchangeable. The mechanism by which *Xenopus* β*TrCP* may suppress the *cdc15* mutation is unknown, but it does not appear to involve the RAS-cAMP pathway.

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ADDENDUM IN PROOF

After submission of this work, the isolation and sequencing of a *Drosophila* cDNA clone encoding the $dTAF_{II}80$ subunit of TFIID and containing seven β-transducin repeats was reported (B. D. Dynlacht, R. O. J. Weinzierl, A. Admon, and R. Tjian, Nature [London] **363**:176–179, 1993). This protein and βTrcp share 24% identity and 51% similarity within the β-transducin repeats; the N-terminal domains are not related.

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