MOLECULAR AND CELLULAR BIOLOGY, June 1989, p. 2715–2723
0270-7306/89/062715-09$02.00/0

Vol. 9, No. 6

Cell Size Modulation by *CDC25* and *RAS2* Genes in *Saccharomyces cerevisiae*

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A detailed kinetic analysis of the cell cycle of *cdc25-1*, *RAS2*$^{Val-19}$, or *cdc25-1*/*RAS2*$^{Val-19}$ mutants during exponential growth is presented. At the permissive temperature (24°C), *cdc25-1* cells show a longer G1/unbudded phase of the cell cycle and have a smaller critical cell size required for budding without changing the growth rate in comparison to an isogenic wild type. The *RAS2*$^{Val-19}$ mutation efficiently suppresses the *ts* growth defect of the *cdc25-1* mutant at 36°C and the increase of G1 phase at 24°C. Moreover, it causes a marked increase of the critical cell mass required to enter into a new cell division cycle compared with that of the wild type. Since the critical cell mass is physiologically modulated by nutritional conditions, we have also studied the behavior of these mutants in different media. The increase in cell size caused by the *RAS2*$^{Val-19}$ mutation is evident in all tested growth conditions, while the effect of *cdc25-1* is apparently more pronounced in rich culture media. CDC25 and RAS2 gene products have been showed to control cell growth by regulating the cyclic AMP metabolic pathway. Experimental evidence reported herein suggests that the modulation of the critical cell size by CDC25 and RAS2 may involve adenylate cyclase.

The budding yeast *Saccharomyces cerevisiae* is a microorganism used as a model eucaryote for the study of the regulation of cell cycle and of cell growth. *S. cerevisiae* has a major cell cycle control point in the regulatory area called start, located in G1. At start, a yeast cell integrates many intracellular and environmental signals and then is committed to continue proliferation or is switched to differentiation pathways such as sporulation, conjugation, or entry into the stationary phase (36).

At start, a critical cell size for budding and for entering the S phase is required (9, 20); this requirement allows for the coordination between growth, which is normally the rate-limiting process in most physiological conditions (1, 20), and nuclear division cycle events. The G1 phase is delayed until this critical mass is reached, which determines a major control mechanism of cell cycle progression (1, 16, 24, 48). The critical cell mass required for budding is strictly related to the nutritional conditions of the cells. Indeed, poor media and low growth rates correspond to smaller critical sizes until a minimum size for budding has been reached and it cannot be further reduced at longer generation times (19, 25, 47, 48).

Several temperature-sensitive (Ts) mutants that arrest the cell division cycle at or near start have been isolated and characterized (15), and two kinds of mutants have been defined on the basis of their terminal phenotypes (38). Class I start mutants (*cdc28*, *cdc36*, etc.) resemble α-factor-treated cells at the restrictive temperature and continue cytoplasmic growth. In class II start mutants (*cdc25*, *cdc33*, *cdc35*), a growth lesion is also apparent; these mutants arrest the cell cycle, mimicking stationary-phase cells (15, 30).

Recently, it has been shown that at least two of the class II start genes, i.e., *CDC25* and *CDC35*, take part in a regulatory mechanism that modulates the cyclic AMP (cAMP) level, which in turn regulates a protein kinase cascade. The *CDC35* gene has been cloned and found to be the structural gene for adenylate cyclase (4, 10), while genetic and molecular data suggest that the *CDC25* gene product may be a modulator of the adenylate cyclase acting together with two proteins, encoded by *RAS1* and *RAS2* genes (5, 7, 39, 46), that share extensive homology with the mammalian *ras* proteins (21). A mechanism mediated by the activation of cAMP-dependent protein kinases positively regulates the cell growth and inhibits differentiating pathways associated with nutrient depletion, such as sporulation or entrance into the G0 phase (33). In fact, all the mutations that inactivate this mechanism, thereby reducing the ability to increase intracellular cAMP levels (*cdc25*, *cdc35*, *ras1*, *ras2*), arrest growth in a manner that mimics nutrient starvation (17, 22, 30). If the inactivation is only partial, as in some *ras2* mutants, it causes a so-called repressed phenotype characterized by high heat shock resistance, hyperaccumulation of glycogen, and inability to grow on nonfermentable carbon sources (44). On the other hand, mutations that constitutively increase cAMP level, such as *CRI4* (13), *SRA4* (8), and *RAS2*$^{Val-19}$ (46), or cause an unregulated protein kinase activity, such as *bcy1-1* (32) and *sra1* (8), generate a derepressed phenotype characterized by the inability to arrest growth in nutrient starvation, the lack of glycogen accumulation, and hypersensitivity to heat shock.

We have previously cloned the *CDC25* gene (2, 28), and we have used the cloned sequence to construct different isogenic strains in order to study the interactions between the *CDC25* and *RAS2* gene products. In this paper, we have shown that the mutation of *CDC25* and *RAS2* genes can alter the yeast cell size by changing the protein content of mother cells at budding. This finding indicates that there may be a direct relationship between the two upstream controlling elements of the cAMP metabolism and the regulation of cell cycle progression at start. Preliminary results are also reported on the possible role of adenylate cyclase and cAMP-dependent protein kinases mediating effects of *CDC25* and *RAS2* on cell size.

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TABLE 1. Yeast strains used in these studies

Strain	Genotype or description	Source or reference
GRF18	*MATα his3-11,15 leu2-3,112*	A. Hinnen[a]
6251-7H1	*MATa lys7 met6*	S. Sora[b]
GH-1	Diploid strain formed by mating GRF18 and 6251-7H1	
NL4	*MATa his3-11,15 leu2-3,112 met6*	Segregant from the GH-1 diploid
NG18	Diploid strain formed by mating NL4 and GRF18	
NG18-D2	*MATa/MATα his3/his3 leu2/leu2 met6/+ cdc25-Δ2::LEU2/+*	Transformant of NG18 with p*CDC25-Δ2::LEU2* cut by *Hin*dIII
1A	*MATα his3-11,15 leu2-3,112 ade2 lys2 tyr1 cdc25-1*	28
NA1	Diploid strain formed by mating NL4 and 1A	
NA1-D2	*MATa/MATα his3/his3 leu2/leu2 ade2/+ lys2/+ tyr1/+ met6/+ cdc25-1/cdc25-Δ2::LEU2*	Transformant of NA1 with p*CDC25-Δ2::LEU2* cut by *Hin*dIII
CNA1-D2	*MATa/MATα his3/his3 leu2/leu2 ade2/+ lys2/+ tyr1/+ met6/+ cdc25-Δ2::LEU2/+*	Transformant of NA1 with p*CDC25-Δ2::LEU2* cut by *Hin*dIII
VNA1-D2	*MATa/MATα his3/his3::pHIS3-RAS2*$^{Val-19}$ *leu2/leu2 ade2/+ lys2/+ tyr1/+ met6/+ cdc25-1/cdc25-Δ2::LEU2*	Transformant of NA1-D2 with *Xho*I-linearized p*HIS3-RAS2*$^{Val-19}$
CVNA1-D2A	*MATa/MATα his3/his3::pHIS3-RAS2 leu2/leu2 ade2/+ lys2/+ tyr1/+ met6/+ cdc25-1/cdc25-Δ2::LEU2*	Transformant of NA1-D2A with *Xho*I-linearized p*HIS3-RAS2*
CVNA1-D2B	*MATa/MATα his3/his3::pHIS3-RAS2* (3 to 5 copies) *leu2/leu2 ade2/+ lys2/+ tyr1/+ met6/+ cdc25-1/cdc25-Δ2::LEU2*	Transformant of NA1-D2A with *Xho*I-linearized p*HIS3-RAS2*
VNA1-1	*MATa/MATα his3/his3::pHIS3-RAS2*$^{Val-19}$ *leu2/leu2 ade2/+ lys2/+ tyr1/+ met6/+ cdc25-1/+*	Transformant of NA1 with *Xho*I-linearized p*HIS3-RAS2*$^{Val-19}$
A364A	*MATa ade1 ade2 ura1 his7 lys2 tyr1 gal4*	YGSC[c]
321	*MATa ade1 ade2 ura1 his7 lys2 tyr1 gal1 cdc25-1*	YGSC
TC7	*MATa ura3 trp1 ade lys5 cdc25-1*	28
T1A	Diploid strain formed by mating 1A and TC7	
N16	*Matα leu2-3,112 ura3 trp1 ade tyr1*	Segregant from the T1A diploid
VN16-P1	*Matα leu2-3,112 ura3 trp1 ade tyr1* p*RAS2*$^{Ala-18\ Val-19}$	Transformant of N16 with p*RAS2*$^{Ala-18\ Val-19}$ plasmid
CVN16-P2	*MATα leu2-3,112 ura3 trp1 ade tyr1* p*RAS2*	Transformant of N16 with p*RAS2* plasmid
N16*	Clone obtained from VN16-P1 after loss of p*RAS2*$^{Ala-18\ Val-19}$ plasmid	
JC302-26B	*MATα his4 leu2 ura3 ras2-530::LEU2*	K. Tatchell[d]
JC303-54	*MATα his4 leu2 ura3 ras2-530::LEU2 SRA4-6*	K. Tatchell
JC303-68	*MATα his4 leu2 ura3 ras2-530::LEU2 sra1-12*	K. Tatchell

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MATERIALS AND METHODS

Strains, media, and growth conditions. *S. cerevisiae* strains used are listed in Table 1. *Escherichia coli* HB101 was used for plasmid amplification and isolation. Media used for yeast cultivation were YEPD (1% yeast extract, 2% peptone, 2% glucose), YNBG (0.67% yeast nitrogen base without amino acids [Difco Laboratories] supplemented with all the auxotrophic requirements [50 mg/l] and 2% glucose), and YM1 (0.5% yeast extract, 1% peptone, 0.67% yeast nitrogen base without amino acids, 1% succinic acid, 0.3% NaOH, buffered at pH 5.8) supplemented with 2% glucose (YMG), 2% raffinose (YMR), or 3% glycerol and 1% ethanol (YMGE). Plates contained 2% agar. Standard yeast genetic methods were followed for strain construction (40). Cells were grown in batch cultures in a shaking water bath at the indicated temperatures. Growth was monitored as the increase in cell number, determined with a Coulter Counter ZBI (Coulter Electronics, Inc.) (48). The percentage of budded cells was determined by microscopic counting on at least 400 cells fixed in 4% Formalin and mildly sonicated (48).

Plasmids and recombinant DNA. Standard recombinant DNA manipulations were performed by the method of Maniatis et al. (26). Enzymes were purchased from Boehringer Mannheim and used in conditions recommended by the supplier. Most of the plasmids used in this work have been already described: p*HIS3-RAS2* and p*HIS3-RAS2*$^{Val-19}$ (22) were obtained by O. Fasano; p*RAS2* and p*RAS2*$^{Ala-18\ Val-19}$ (39) were obtained by K. Tatchell; p*cdc25*SUP/2 (23) was obtained from J. Lisziewicz. p*cdc25-Δ2::LEU2* was constructed as follows. The pDGEm-1 plasmid (28) was linearized with *Bam*HI and partially digested with *Bgl*II. The 7.9-kilobase (kb) fragment carrying a 2.6-kb *Bgl*II-*Bam*HI deletion of the *CDC25* open reading frame was purified by preparative agarose gel electrophoresis and ligated with a 3-kb *Bgl*II-*Bgl*II fragment, derived from YEp13, containing the *LEU2* gene. The LiCl method of Ito et al. (18) was used for transformation of *S. cerevisiae*. Quick preparation of yeast DNA was performed by the method of Crabeel et al. (11). Southern blot hybridization was performed as described by Maniatis et al. (26). Labeled probes were prepared by nick translation of appropriate DNA fragments.

Disruption of *CDC25*. A 5-kb *Hin*dIII fragment of p*cdc25-Δ2::LEU2* was used to transform NA1 and NG18 diploid yeast cells, and transformants were selected by leucine prototrophy. The integration events were mapped by Southern blot analysis. Tetrad analysis of disrupted strains showed a 2:2 (viable:nonviable) ratio of spores. The viable spores were always *leu* mutants, and those derived from NA1-D2 strain were Ts *leu* mutants.

Analysis of yeast population parameters. Mean cell volume was estimated from the cell volume distributions obtained by the Coulter Channelyzer, calibrated with 8.79-μm latex

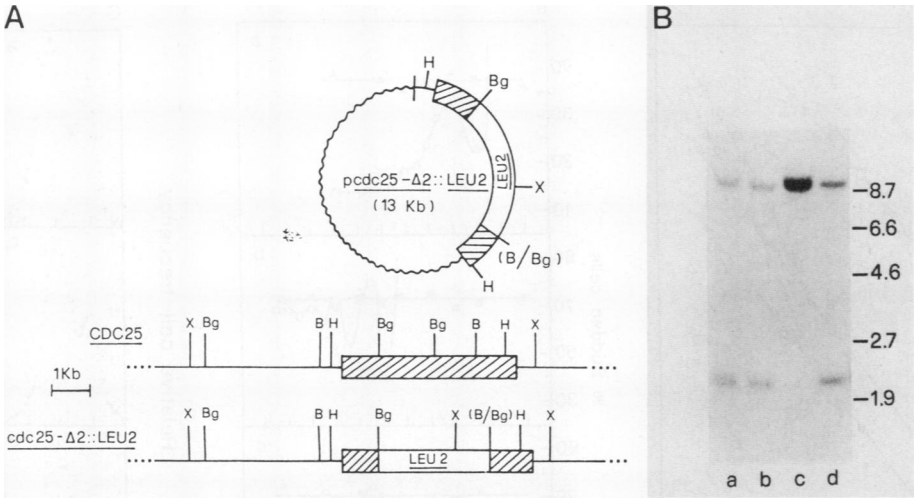

FIG. 1. *CDC25* gene disruption. (A) The p*CDC25-Δ2::LEU2* plasmid cut by *Hin*dIII was used to perform the *CDC25* gene disruption that is schematically shown in the lower part of the figure representing the wild-type and the disrupted *CDC25* locus. In the disrupted *CDC25* gene, the *Bgl*II-*Bgl*II-*Bam*HI internal fragment of the *CDC25* open reading frame was replaced with the *LEU2*-containing *Bgl*II-*Bgl*II DNA fragment. (B) Southern blot analysis of genomic DNA extracted from NA1-D2 (lane a), CNA1-D2 (lane b), NA1 (lane c), and NG18-D2 diploid (lane d) strains and cut with *Xho*I. A ^{32}P-labeled *CDC25* probe spanning the C-terminal half of the gene was used. The results demonstrate a heterozygous gene disruption marked by the new 2.4-kb hybridization band present together with the wild-type band. ▨, *CDC25* open reading frame; ▭, *LEU2*-containing DNA fragment; ——, chromosomal sequences near the *CDC25* open reading frame; ∿∿, YRp7 vector sequence. B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; X, *Xho*I. Note that we have not mapped all the possible *Hin*dIII sites that could be in the *Xho*I-*Xho*I fragment containing the *CDC25* gene.

beads by using the formula $\overline{V} = (\Sigma_{n_i} \cdot c_i/\Sigma_{n_i})F$, where n_i is the number of cells contained in the c_i channel and F is a calibration factor (48). The distributions were acquired and analyzed with a MINC 11/23 computer (Digital Equipment Corp.).

Flow cytometric analysis on the yeast population was performed with a FACSIV cytofluorometer (Becton Dickinson and Co.) after staining with propidium iodide for DNA and fluorescein isothiocyanate for protein (48). About 10^5 cells were scored for each distribution, and the histograms were stored on floppy disk and further analyzed with a MINC 11/23 computer. DNA distribution were analyzed with an algorithm based on the method of Slater et al. (41). Protein distributions were analyzed with a program developed in our laboratory (3, 31) that determines a fit of experimental protein distributions: the temporal parameters T_P, mother cell generation time, and T_D, daughter cell generation time, starting from the experimental distribution; and the population doubling time expressed in minutes T [$T = (\ln 2)/k \cdot 60$], where k (h^{-1}) is the constant of exponential growth (3).

The length of budded phase, T_B, was derived from the fraction of budded cells (F_B) by $T_B = \log_2(1 + F_B)T$. P_s, the average protein content of a mother cell at budding, was calculated by the method of Vanoni et al. (48): $P_s = P_p \exp[k\ (T_P - T_D)/60]$, where P_p is the average protein content of a parent cell at the beginning of a cycle and was calculated by the method of Vanoni et al. (48); $P_p = (60 \cdot \overline{P})/k\ [T_P - T_D + T_D \exp(K \cdot T_P/60)]$, where $\overline{P}$ is the mean protein content, derived from protein distribution as the mean fluorescence channel and converted to picograms per cell by using a reference distribution. The reference distribution was obtained by analyzing, during the same acquisition round, the distribution of S288C strain exponentially growing in YEPD medium and having an average of 3.8 pg of protein per cell (37, 48).

The volume of mother cells at budding (V_B) was obtained by measurement of the lengths of major and minor axes of suitable cells on microphotographs and considering a yeast cell as an approximate prolate spheroid (42). Cells were mildly sonicated, fixed with 4% Formalin, and stained with Calcofluor (48). Stained cells were photographed with a Leitz Dialux fluorescence microscope by using Ilford FP4 film.

RESULTS

Suppression of the growth defects caused by a *cdc25* mutation by an activated *RAS2* gene. A heterozygous *cdc25-1*/*CDC25* diploid strain (NA1) was constructed (Table 1). A gene disruption (Fig. 1A) of the wild-type copy of the *CDC25* gene was then performed as demonstrated by a Southern blot analysis (Fig. 1B) and by the expected segregation of the *ts* phenotype (*cdc25-1* is recessive). The subsequent NA1-D2 *ts* strain was then integratively transformed either with p*HIS3-RAS2* or with p*HIS3-RAS2*$^{Val\text{-}19}$ (22). The single copy of the *RAS2*$^{Val\text{-}19}$ allele present in the isogenic VNA1-D2 strain confers to the cells, in a dominant way, the ability to grow at restrictive temperature while one or even several copies of the wild-type *RAS2* gene (CVNA1-D2A and CVNA1-D2B strains) were ineffective. These data are in agreement with a proposed functional interaction between the two gene products and confirm similar findings obtained in other laboratories (5, 39).

To investigate in more detail the correlation between the genetic setup and the cell cycle control, we performed a detailed analysis of cell cycle and the growth parameters of *cdc25-1* and *RAS2*$^{Val\text{-}19}$ mutants. For this purpose, the NA1 strain and the isogenically derived NA1-D2 and VNA1-D2 strains were exponentially grown in YEPD liquid medium at 24°C and then shifted to 36°C while several relevant kinetic parameters of the cell cycle, such as the growth rate, the fraction of budded cells, and DNA and protein distribution obtained by flow cytometry, were monitored. At the permis-

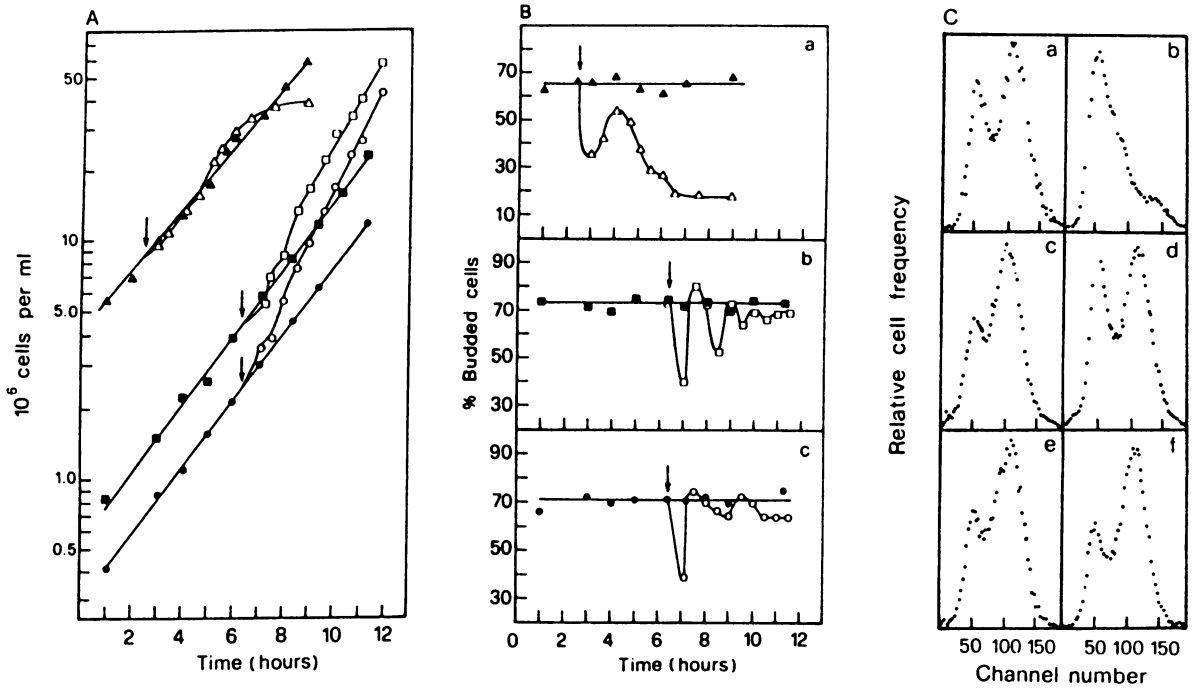

FIG. 2. Effect of a temperature shift on growth and cell division in NA1, NA1-D2, and VNA1-D2 strains. Exponentially growing cells in YEPD medium at 24°C were shifted at 36°C when indicated by arrows. (A) Cell number increase in NA1 (●, ○), NA1-D2 (▲, △), and VNA1-D2 (■, □) strains at 24°C (closed symbols) and at 36°C (open symbols). (B) Percent of budded cells in NA1 (c), NA1-D2 (a), and VNA1-D2 (b) strains at 24°C (closed symbols) and at 36°C (open symbols). (C) Cellular DNA content frequency distributions at 24°C (a, c, and e) and after 5 h at 36°C (b, d, and f) in NA1-D2 (a and b), NA1 (c and d), and VNA1-D2 (e and f) strains.

sive temperature, no significant differences were observed between the generation times of all the strains (Fig. 2A; Table 2). When shifted to 36°C, the three strains behaved very differently. The NA1-D2 Ts strain underwent an arrest with biphasic kinetics in the unbudded G1 phase of the cycle (Fig. 2A to C) as previously shown for this start mutant by using a synthetic medium (29, 30). On the other hand, the VNA1-D2 strain gave a response to the temperature shift which was identical to that of the wild type, demonstrating that the *RAS2*$^{Val-19}$ mutation can efficiently suppress the impaired *CDC25* function in the Ts mutants. The phenotypes of the control strains CNA1-D2 (derived from NA1 by gene disruption of the mutated copy of *CDC25*) and CVNA1-D2A or -B were not distinguishable from those of the NA1 and NA1-D2 strains, respectively (data not shown). This efficient suppression of the Ts growth phenotype is in accordance with a regulation of cell cycle progression by the *RAS2*$^{Val-19}$ that is no longer dependent on the *CDC25* function.

It is worth noting that there was a reproducible small increase in the relative duration of the G1 and unbudded phases of the cycle of the *cdc25-1* mutant at 24°C in comparison with that of the wild-type strain. This was calculated by using the F_{G1} and F_B experimental values in the different populations (Fig. 2B and 2C; Table 2). This

TABLE 2. Temporal parameters of yeast populations

Medium	Strain	Relevant genotype	Parameter result[a]					
			T	F_B	T_B	F_{G1}	T_P	T_D
YEPD	NA1	*cdc25-1/CDC25*	135	0.75	109	0.24	128	150
	NA1-D2	*cdc25-1/cdc25-Δ2::LEU2*	138	0.65	100	0.34	119	169
	CNA1-D2	*cdc25-Δ2::LEU2/CDC25*	136	0.74	109	0.24	128	152
	VNA1-D2	*cdc25-1/cdc25-Δ2::LEU2 his3/his3::pHIS3-RAS2*$^{Val-19}$	132	0.73	105	0.27	122	150
	CVNA1-D2	*cdc25-1/cdc25-Δ2::LEU2 his3/his3::pHIS3-RAS2*	140	0.67	104	0.32	123	167
	A364A	Wild type	132	0.68	99	ND	123	153
	321	*cdc25-1*	130	0.60	89	ND	112	164
YNBG	NA1	*cdc25-1/CDC25*	234	0.65	169	0.32	211	281
	NA1-D2	*cdc25-1/cdc25-Δ2::LEU2*	222	0.45	119	0.55	157	337
	VNA1-D2	*cdc25-1/cdc25-Δ2::LEU2 his3::his3::pHIS3-RAS2*$^{Val-19}$	228	0.66	167	0.32	208	271
	N16	*cdc25-1*	186	0.50	109	ND	142	264
	VN16	*cdc25-1* p*RAS2*$^{Ala-18\ Val-19}$	378	0.46	205	ND	308	501
	CVN16	*cdc25-1* p*RAS2*	192	0.56	122	ND	156	255
	N16*	*cdc25-1*	185	0.51	108	ND	145	263

[a] F_{G1}, Fraction of cells in G1 phase; other parameters are defined in Materials and Methods. T, F_B, and F_{G1} are experimentally determined, while T_B, T_P, and T_D are calculated as described in the text. ND, Not determined.

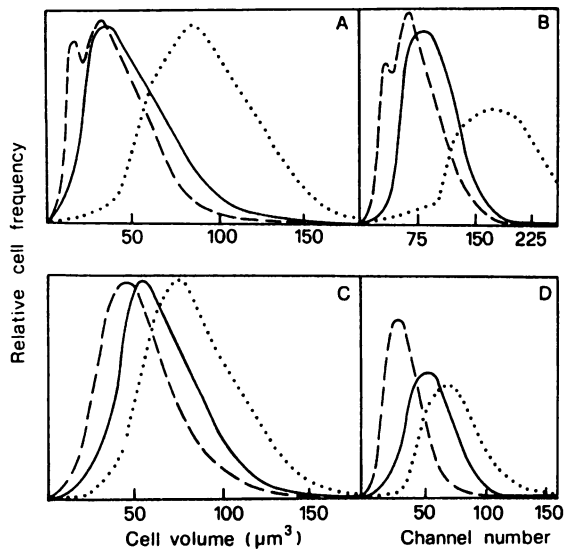

FIG. 3. Cell volume and protein frequency distributions of mutant strains. NA1 (——), NA1-D2 (– – –), and VNA1-D2 (·····) strains were exponentially grown in YNBG (A and B) or YEPD (C and D) medium at 24°C. (A and C) Cell volume distributions obtained with a Coulter Counter-Channelyzer. (B and D) Protein content distributions obtained by flow cytometry. Channel scale of the panel B distributions is amplified 2.1× in comparison with distributions in panel D.

phenomenon was much more evident in minimal media like YNBG (Table 2), where it was coupled with an exceptionally high heat shock resistance (about 100 times greater than that of an isogenic wild type), as shown by the *cdc25-1* strains (29; unpublished results). In particular, the daughter generation time (T_D) is greatly increased as a consequence of a more asymmetrical division. This G1 phenotype could be due to a partial expression of the *cdc25-1* mutation also at 24°C, and again it was fully reverted in the double mutant VNA1-D2, in which the *RAS2*$^{Val-19}$ mutation was also present (Fig. 2B and C; Table 2). Moreover, the distributions of the cellular DNA content and the frequency of budded cells indicated that *RAS2*$^{Val-19}$, at least in the presence of the *cdc25-1* mutation, did not affect the timing of the cell cycle during growth at 24°C in YEPD or YNBG medium.

Modification of the cell size distribution of yeast populations by *cdc25-1* and *RAS2*$^{Val-19}$ mutations. Cell volume and protein content distributions taken during exponential growth at 24°C were different for the three tested strains. As shown in Fig. 3, the average cell volume and protein content per cell decreased in the *cdc25* Ts mutant while the strain containing the *RAS2*$^{Val-19}$ allele had a higher volume and a higher protein content in comparison with the wild-type strain (Fig. 3; Table 3). Control strains containing the *RAS2* gene integrated at the *HIS3* locus in both single copy and multicopies did not show any change in cell size distribution, while the presence of a *RAS2*$^{Val-19}$ gene increased the cell size even when a wild-type *CDC25* gene (strain VNA1-1) was present.

Since the NA1-D2 strain was constructed by gene disruption of one copy of the *CDC25* gene of the diploid parental strain, it was important to verify whether the small phenotype of NA1-D2 strain was due to a gene dosage effect. However, we can exclude this possibility since the CNA1-D2 strain, which retained only the wild-type copy of the *CDC25* gene, does not have a phenotype distinguishable from that of NA1. Moreover, the same gene disruption was performed on one copy of the *CDC25* gene in the wild-type homozygous (*CDC25/CDC25*) NG18 strain, deriving the NG18-D2 strain (Table 1) and in this case (data not shown) no variation in volume or protein distributions was observed.

It was possible to conclude that a significant decrease in the average cell size of a yeast population was associated with the presence of the *cdc25-1* mutation and that the *RAS2*$^{Val-19}$ mutation acted in the opposite way under the tested conditions. Moreover, the double mutant VNA1-D2 showed an increase of protein content per cell when compared with an isogenic wild type.

The critical cell size required for bud emergence is changed by *RAS2*$^{Val-19}$ and *cdc25-1* mutations. A quantitative analysis of the protein distributions of NA1, NA1-D2, and VNA1-D2

TABLE 3. Growth parameters and critical protein threshold values

Medium	Strain	Relevant genotype	Parameter result[a]			
			$\overline{V}$	$\overline{P}$	P_s	V_B
YEPD	NA1	*cdc25-1/CDC25*	64 (1.00)	6.40 (1.00)	5.14 (1.00)	57.0
	NA1-D2	*cdc25-1/cdc25-Δ2::LEU2*	48 (0.75)	4.35 (0.68)	3.67 (0.71)	37.5
	CNA1-D2	*cdc25-Δ2::LEU2/CDC25*	65 (1.01)	6.30 (0.98)	5.08 (0.99)	ND
	VNA1-D2	*cdc25-1/cdc25-Δ2::LEU2 his3/his3::pHIS3-RAS2*$^{Val-19}$	78 (1.23)	8.36 (1.3)	6.80 (1.32)	70.0
	CVNA1-D2	*cdc25-1/cdc25-Δ2::LEU2 his3/his3::pHIS3-RAS2*	47 (0.73)	4.70 (0.73)	3.96 (0.77)	ND
	A364A	Wild type	32 (1.00)	4.80 (1.00)	3.76 (1.00)	ND
	321	*cdc25-1*	22 (0.68)	2.56 (0.53)	2.20 (0.58)	ND
YNBG	NA1	*cdc25-1/CDC25*	47 (1.00)	4.90 (1.00)	4.10 (1.00)	39.0
	NA1-D2	*cdc25-1/cdc25-Δ2::LEU2*	41 (0.87)	3.80 (0.77)	3.71 (0.90)	39.0
	VNA1-D2	*cdc25-1/cdc25-Δ2::LEU2 his3/his3::pHIS3-RAS2*$^{Val-19}$	92 (1.96)	9.40 (1.92)	7.80 (1.90)	81.5
	N16	*cdc25-1*	20 (1.00)	1.84 (1.00)	1.72 (1.00)	ND
	VN16	*cdc25-1* p*RAS2*$^{Ala-18\ Val-19}$	41 (2.06)	3.52 (1.91)	3.36 (1.95)	ND
	CVN16	*cdc25-1* p*RAS2*	25 (1.24)	2.26 (1.23)	2.02 (1.17)	ND
	N16*	*cdc25-1*	21 (1.05)	1.83 (0.99)	1.70 (0.98)	ND

[a] $\overline{V}$ is the average cell volume expressed in cubic micrometers; $\overline{P}$ is the average cell protein content expressed in picograms per cell; P_s is the critical protein threshold required for budding; V_B is the average critical cell size required for budding expressed in cubic micrometers. $\overline{V}$, $\overline{P}$, and V_B are experimentally determined, and P_s is calculated by using a structured model of yeast population as described in the text. Normalized values of the various parameters are reported in parentheses; they are normalized to a wild-type value of 1 except for VN16-P1, CVN16-P2, and N16* strains, whose values are normalized to the value for the *cdc25-1* N16 strain. ND, Not determined.

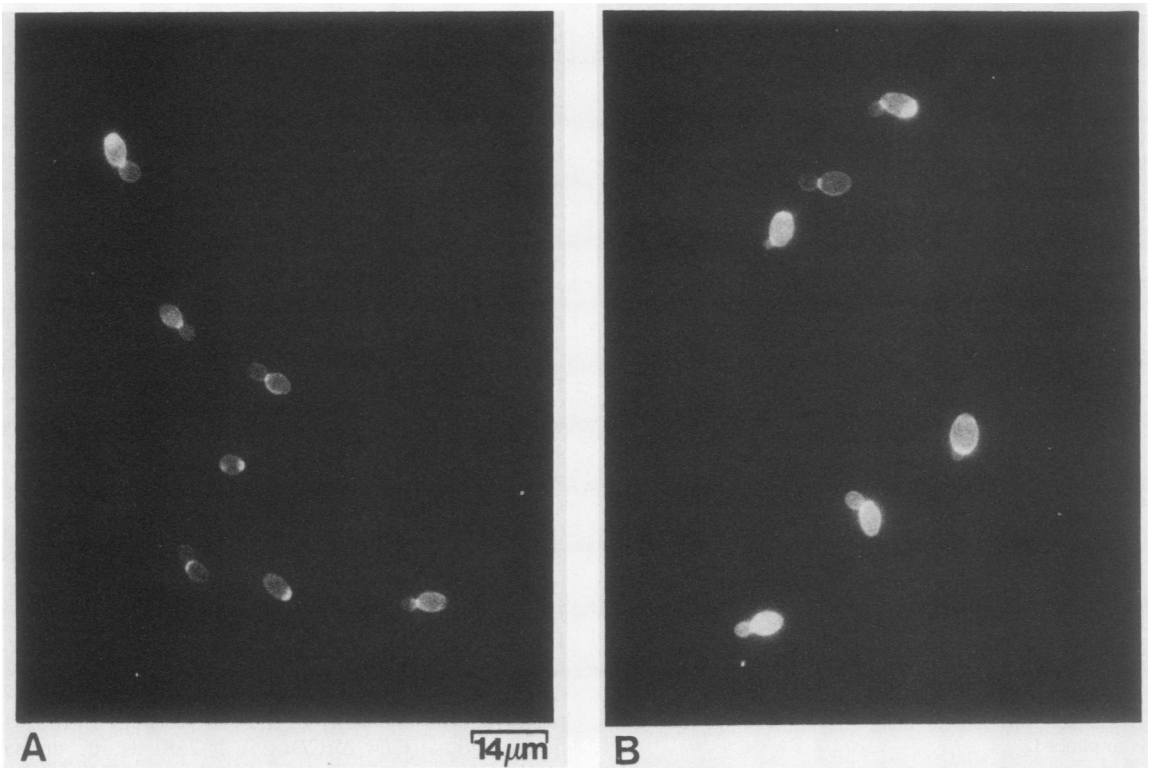

FIG. 4. Microphotographs of mutant cells. Calcofluor-stained cells of NA1-D2 (A) and VNA1-D2 (B) strains were photographed under UV light with a fluorescence microscope with a 63× objective.

strains growing in YEPD medium was then performed by means of a structured model developed in our laboratory which describes a yeast population in balanced exponential growth (3). Using a computer program based on such a model (31), we determined the temporal parameters of the populations of NA1, NA1-D2, and VNA1-D2 strains growing exponentially at 24°C in YEPD medium (Table 2) and the protein content of mother cells at budding (P_s). As shown in Table 3, the *cdc25-1* mutation lowered the critical protein content required for budding (P_s) and the *RAS2*$^{Val-19}$ corrected such a phenotype. Moreover, in strain VNA1-D2, *RAS2*$^{Val-19}$ elevated this critical protein threshold in comparison with the value shown by an isogenic wild type.

We also performed a direct measurement of the size of mother cells by taking a series of microphotographs of fixed cell samples of NA1, NA1-D2, and VNA1-D2 strains exponentially growing on YEPD medium at 24°C. A direct determination of the volume of at least 100 mother cells with small buds was then performed by assuming the cell shape as a prolate spheroid and by measuring the maximum and minimum diameters of the cells on projections of the photographic films. These values can be assumed to be very close to the critical cell size required for budding (V_B) because the growth in size occurs in the bud after a mother cell has budded (19, 25, 36).

The value of V_B was decreased for NA1-D2 and increased for VNA1-D2 in comparison with that of the isogenic wild type (Table 3), which was in accordance with the corresponding variation of the calculated P_s values. The microphotographs (Fig. 4) point out the striking difference between the V_B values of strains NA1-D2 and VNA1-D2 (about 85%). We chose to use isogenic strains for the experiments described here; nevertheless, the modulation of the cell size, related to the presence of the *cdc25-1* and the *RAS2*$^{Val-19}$ alleles, might be dependent upon the genetic background of the strains.

We then compared strain 321, in which the *cdc25-1* mutation was originally isolated, with the parental isogenic A364A strain. These two strains were exponentially grown in YEPD medium. The growth parameters were monitored as previously described, and the P_s value was calculated. The data (Tables 2 and 3) showed a decrease of the P_s value in the mutated strain in comparison with that of the wild type. The difference is even more pronounced than for NA1 and NA1-D2 strains, confirming the role of the *cdc25-1* lesion in modulating yeast cell size.

Another haploid *cdc25-1* strain (called N16) was transformed with the p*RAS2* and p*RAS2*$^{Ala-18\ Val-19}$ centromeric plasmids (39) that contained a wild type or a mutated copy of the *RAS2* gene, respectively, on the YCp50(URA3) vector. In accordance with the findings of Robinson et al. (39), the ts phenotype was reverted in all of the *ura*$^+$ transformants obtained with p*RAS2*$^{Ala-18\ Val-19}$ but not in p*RAS2*-containing clones. Moreover, after growth in nonselective conditions, we also recovered some *ura* mutant clones from a p*RAS2*$^{Ala-18\ Val-19}$ transformant, and each of them also became *ts* for growth.

The growth parameters and the P_s values reported in Tables 2 and 3 were determined for these strains during the exponential growth in YNBG medium. These data demonstrate once again that an increase of the protein content required for budding is specifically associated with the presence of an activated copy of the *RAS2* gene. We have concluded that the described modulation of the size required for entering into the cell division cycle is specifically caused by mutations in the *CDC25* and *RAS2* genes.

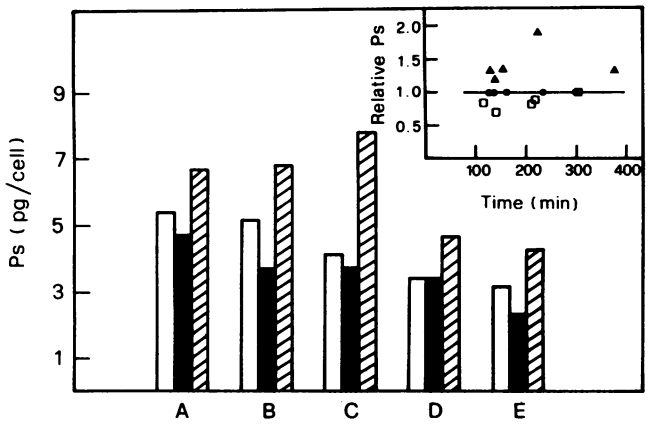

FIG. 5. P_s values of *cdc25-1* and *RAS2*$^{Val-19}$ cells in different growth conditions. The P_s values of NA1 (□), NA1-D2 (■), and VNA1-D2 (▨) cells were calculated by using a structural model of yeast population, as described in the text, during exponential growth in different media. A, YMG; B, YEPD; C, YNBG; D, YMGE; E, YMR. Duplication times (in minutes) were as follows: YMG, 126 (NA1), 117 (NA1-D2), 138 (VNA1-D2); YEPD and YNBG, given in Table 2; YMR, 162 (NA1), 216 (NA1-D2), 150 (VNA1-D2); YMGE, 300 (NA1), 300 (NA1-D2), 378 (VNA1-D2). Insert: P_s values of NA1-D2 (□) and VNA1-D2 (▲) strains were normalized against the NA1 P_s values (●) taken as 1 in any condition and plotted against the duplication times.

Alteration of P_s by *RAS2*$^{Val-19}$ and *cdc25-1* mutations in different growth conditions. The P_s values of the NA1, NA1-D2, and VNA1-D2 cells have been determined in different growth conditions (duplication times ranging from 117 to 380 min) (Fig. 5). As expected, there was a strong dependence of P_s values from the growth rate and the data relative to the wild type were in accordance with those reported for diploid cells (19). Also in Fig. 5, the relative P_s of the mutants was plotted against the mass doubling time after a normalization of the wild-type P_s values set to 1 for each growth condition. The effects related to the *RAS2*$^{Val-19}$ allele were roughly independent of the generation times with the exception of the highest relative P_s and V_B values that were obtained in YNBG medium (Fig. 5 and Table 3). Rather, the best expression of the small phenotype of *cdc25-1* was obtained in rich media, thus suggesting that the relative P_s values of NA1-D2 progressively approximate those of the wild type at increasing generation times.

Possible relationships between yeast cell size control and the cAMP pathway. Biochemical and genetic evidence indicates that *CDC25* and *RAS2* gene products act as two regulatory upstream elements of the cAMP metabolic pathway (5, 39) through a modulation of the activity of the adenylate cyclase. Therefore, the described effect on cell size of *cdc25-1* or *RAS2*$^{Val-19}$ mutations may be mediated by modifications of the cAMP level and, since the only target of cAMP known in yeast cells is the regulatory subunit of the cAMP-dependent protein kinase, by an alteration of the intracellular protein kinase activity. Thus, we expected that an activation of the adenylate cyclase or cAMP-dependent protein kinase might increase the cell size at budding. To investigate this point, we determined the growth kinetic parameters and P_s cellular values of JC302-26B, JC303-54, and JC303-68 isogenic strains (8) (Table 1) that harbor mutations in the cAMP pathway. JC303-68 carries the *sra1* lesion (allelic to *bcy1*) that inactivates the regulatory subunit of cAMP-dependent protein kinase, thus allowing a constitutive unregulated protein kinase activity; JC303-54 carries the *SRA4* mutation that activates adenylate cyclase (43). Both mutations are able to suppress the growth lesion of the cdc25-1 strain as well as of ras1$^-$ ras2$^-$ disrupted strains (43). One can see from Table 4 that the three strains have an almost identical growth rate and fraction of budded cells during exponential growth in YEPD medium at 24°C. The presence of the *SRA4* mutation was associated with a marked increase of the cell size measured as P_s values, while the presence of a *sra1* lesion did not determine a significant size variation of the cells.

Experiments were also performed with the N16 strain transformed with a p*cdc25*SUP/2 plasmid carrying the *PK25* gene (23) also known as TPK1 (45), which codes the catalytic subunit of a cAMP-dependent protein kinase. As previously reported (23), the expression of the *PK25* gene on a multicopy plasmid suppressed the growth lesion of the *cdc25-1* strain (data not shown). As with the *sra1* mutant, no significant increase of critical protein content required for budding was observed in the N16 strain transformed with p*cdc25* SUP/2 in comparison with the isogenic control strain during exponential growth in YNBG selective medium at 24°C (data not shown).

Taken together, these findings offer a preliminary indication of a possible role of cAMP levels or, at least, activated adenylate cyclase in modulating cell size. However, at least in the tested conditions, this effect does not appear to be simply related to the activation of the known yeast protein kinases brought on by the *sra1* mutation or by the overexpression of the *PK25* gene coding for one of these kinases.

DISCUSSION

The data presented in this paper show in detail several aspects of the phenotype of *cdc25-1* and *RAS2*$^{Val-19}$ strains. A single copy of the *RAS2*$^{Val-19}$ gene is sufficient to fully complement all the growth defects caused by the *cdc25-1* mutation at both permissive and restrictive temperatures. Furthermore, cells that bear both mutations are heat shock sensitive and undergo defective growth arrest in nutrient starvation (5; M. D. Baroni, unpublished results). The marked differences in cell size and protein distributions that have been found among the different strains are mainly due to an altered size of mother cells at budding and to a proportional change of the protein content required for budding (P_s). All the strains containing a *cdc25-1* allele that

TABLE 4. Temporal and growth parameters of yeast populations

Strain	Relevant genotype	Parameter result[a]					
		T	F_B	T_B	$\overline{V}$	$\overline{P}$	P_s
JC302-26B	*ras2-530*	138	0.67	102	33 (1.00)	3.5 (1.00)	3.00 (1.00)
JC303-68	*sra1-12 ras2-530*	135	0.67	100	36 (1.09)	3.8 (1.08)	3.24 (1.08)
JC303-54	*SRA4-6 ras2-530*	138	0.69	104	45 (1.35)	5.6 (1.6)	4.64 (1.55)

[a] Parameters are defined in Tables 2 and 3 and Materials and Methods.

have been tested showed a reduced P_s, while strains bearing the $RAS2^{Val-19}$ allele showed, in a dominant way, an increased critical size for budding in comparison with that of an isogenic wild type.

These phenotypes are not dependent on the genetic background of the cells and are also observable in different growth media. More importantly, they could not be explained by nonspecific variations of cell sizes because similar growth rates were observed when the $RAS2^{Val-19}$- or *cdc25-1*-bearing cells were compared with the wild-type strain. Under the same conditions, the relative duration of cell phases was also found to be unaffected in the *cdc25-1* $RAS2^{Val-19}$ double-mutant cells, as determined by DNA content distributions.

Mutations in two genes have been previously shown to alter the cell size in *S. cerevisiae*: the *whi1*-bearing cells bud at a critical cell mass that is about half the size of the corresponding wild type without relevant growth rate modifications, while the *whi2* mutation causes a reduction of cell size only when cells enter into stationary phase (42). A genetic and physiological analysis demonstrated that the *WHI1* gene product acts in a dose-dependent way (42) and may encode for an activator of mitosis (B. Fuchter, personal communication).

Our findings indicate that *cdc25-1* and $RAS2^{Val-19}$ are also true cell size mutations. In fact, they are able to modify the critical size of cells without any effect on culture doubling times. The decrease of P_s values caused by the *cdc25-1* mutation was noticeably less evident under poor growth conditions and there were no differences found with the wild type in either YM-1–acetate medium (data not shown) or in YMGE. This finding could be explained if the signal altered by *cdc25-1* mutation was no longer necessary under these conditions; however, it would be necessary in order to increase the cell size in rich media and it appears constitutively activated in $RAS2^{Val-19}$ cells.

The *CDC25* gene product is considered a molecular detector of nutrient availability of cells whose signals are apparently transduced by *ras* proteins (2, 5, 6, 12, 14, 27, 34, 39). When this system is genetically altered by *cdc25-1* and $RAS2^{Val-19}$ mutations, two main effects are observed under very different conditions: a constitutive repression or derepression of cell growth and metabolism that is no longer dependent on the actual nutritional conditions of cells (30, 46) and an altered modulation of critical cell size during the exponential phase of growth, as demonstrated in this paper. Both phenotypes are probably caused by altered nutritional information given to cells by the *CDC25-RAS2*-modified system. However, it should be stressed that in the second case a control of the cell cycle progression that is exerted by *CDC25* and *RAS2* elements is also apparent.

This finding prompts three related questions: what is the signal regulating nutritional cell size modulation and affected by *cdc25-1* and $RAS2^{Val-19}$ mutations? Is it the same signal regulating cell growth, which is the cAMP regulative cascade? And finally, how does the signal interact with the molecular mechanism that actually determines the critical cell size requirement? In response to the second question, we began investigation on the phenotype of adenylate cyclase and cAMP-dependent protein kinase mutants. We actually found that a 55% increase of protein content at budding is associated to the *SRA4* mutation, while a *sra1* lesion or the overexpression of the *TPK1* gene does not modify cell size. Nevertheless, under the tested conditions, the same mutations can derepress cell growth in nutrient starvation and suppress the *cdc25-1* phenotype (8, 23, 43, 45; data not shown).

Several laboratories have measured the intracellular cAMP level variations that are determined by the mutations considered in this study. They have also found very consistent values in different genetic backgrounds. From these data, it is quite evident that higher cAMP levels of $RAS2^{Val-19}$-bearing strains (46) are correlated with a cell size increase. However, the lower P_s value observed in *cdc25-1* allele-bearing cells does not correspond to a decreased cAMP intracellular concentration. In fact, in contrast to the phenotype of *cdc25-5*-bearing cells, incubation of cells containing the *cdc25-1* allele at restrictive conditions does not decrease cAMP level (29, 35), nor does it impair the activation in vivo of the adenylate cyclase exerted by glucose (35; L. Van Aelst and J. M. Thevelin, personal communication). These data can be explained if a pathway different from cAMP-protein kinase A cascade is responsible for the described P_s modulations. This pathway should be regulated by *CDC25* and *RAS2* gene products and should also be able to interact with the adenylate cyclase. Alternatively, the critical size variations might be mediated by a cAMP-dependent protein kinase different from *TPK1* (45).

ACKNOWLEDGMENTS

This work was partially supported by a C.N.R. Grant Target Project, "Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie," no. CT(6.00092.51.115.16078.) to E.M. and by an MPI 40% Grant (1986) to L.A.

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