[CANCER RESEARCH 53, 1867–1870, April 15, 1993]

Yeast Cells Can Enter a Quiescent State through G_1, S, G_2, or M Phase of the Cell Cycle[1]

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ABSTRACT

We have examined the ability of the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* to enter a quiescent state through G_1, S, G_2, or M phase of the cell cycle. We monitored entry to a quiescent state by measuring two well known properties of quiescent cells, *i.e.*, long-term viability and a dramatic increase in resistance to thermal heat shock relative to cycling cells. For this purpose, we made use of yeast cell division cycle (*cdc*) mutants with which we could arrest most of the cells in culture at specific points in the cell cycle. We find that these eukaryotes can enter a reversible quiescent state at any of the points in the cell cycle we examined if the cells are exposed to starvation conditions (starvation normally signals cells to leave the cell cycle). These findings indicate that mechanisms involved in entry to and exit from a quiescent state can operate not only in G_1 phase (leading to G_0 arrested cells) but can also operate in S, G_2, and M phases of the cell cycle. These findings may be important for clinical oncology in cases where tumor cells escape the cytotoxic effects of chemotherapeutic agents. It may be that escape from the effect of these drugs is due to tumor cells entering quiescent states at points in the cell cycle other than G_1 phase. Perhaps different chemotherapeutic strategies may be required to kill tumor cells reentering the cell cycle from other than G_1.

INTRODUCTION

Eukaryotic cells do not divide continuously. Under certain environmental conditions they stop cell division, leave the cell cycle, and enter a stable quiescent state (1, 2). Quiescent cells remain viable for extended periods of time and are much more resistant to thermal heat shock than actively cycling cells (1). For most eukaryotic cells, environmental signals which cause growth arrest result in cells arrested in the G_1 phase of the cell cycle. These arrested cells enter a quiescent state often referred to as G_0 (1, 2). A notable exception to this is found in the yeast *Schizosaccharomyces pombe*, where entry into a quiescent state occurs from either G_1 or G_2 (3, 4). It remains unclear what mechanisms are involved in entry to quiescence. Furthermore, it is unclear whether the mechanisms which cause entry into quiescence can function only at specific points in the cell cycle, *i.e.*, via G_1 for most eukaryotic cells and via G_1 or G_2 for *Sch. pombe* cells. Previous work has shown that a small number of mammalian cells in culture do not enter a quiescent state in G_1 but appear to enter an SQ[3] phase or quiescent state in G_2 phase and that these quiescent states are reversible (5). Because only a small number of cells in these experiments was characterized as entering an SQ phase or a quiescent state in G_2 it remains uncertain whether entry into quiescence can in general occur at various points in the cell cycle. For this purpose we made use of a number of *Sch. pombe* and *Saccharomyces cerevisiae* temperature-sensitive *cdc* mutants which arrest in either G_1, S, G_2, or M phase of the cell cycle (6, 7). Although these cells are arrested at specific points in the cell cycle they do not undergo growth arrest; consequently they continue to increase their mass. By this method we can arrest a large percentage of cells in a population at a specific point in the cell cycle and examine their ability to become quiescent. After arresting these mutants in the cell cycle, we starved the cells for nutrients, which normally causes growth arrest and entry into quiescence. Yeast cells which have entered a quiescent state are much more resistant to thermal heat shock than actively cycling cells. Furthermore, quiescent cells are viable for long periods of time in the absence of nutrients. Therefore, we assayed for quiescence by long-term viability and resistance to thermal heat shock. We find the position at which yeast cells can enter quiescence is not limited to G_1 or G_2 but can occur at any of the *cdc* arrest points we examined.

MATERIALS AND METHODS

Yeast Strains. The *Sch. pombe* strains used here are shown in Table 1. The *S. cerevisiae* cell division cycle mutant strains used here were kindly provided by John Nitiss, Kelly Tatchell, and Michael Wigler. Strain SP1 has been previously described (8).

Culture Conditions for Yeast Strains. *Sch. pombe* culture conditions and heat shock conditions were carried out as described in the text. Details of culture medium and culture conditions have previously been described (9). Hydroxyurea-containing medium was supplemented with 12 mM HU as previously described (9). The *S. cerevisiae* culture conditions were carried out as described in the text. YPD medium was used as a rich medium and starvation medium was identical to YPD medium with the exception of reduction of the glucose concentration from 20 g/liter to 0.10 g/liter (10). Heat shock conditions for the *S. cerevisiae* strains were carried out as described in the text. Cell numbers present in *Sch. pombe* cultures were determined by using a Coulter Counter (11). Cell numbers for *S. cerevisiae* cultures were estimated from determination of A_{600} where an $A_{600} = 1$ is equivalent to 1×10^7 cells/ml. For calculation of percentage of heat shock resistance and percentage of survival after long-term starvation, we use a standard formula. Because we were working with large numbers of cells, and because we made serial dilutions before plating the cells, the numbers in the denominator of the formula to calculate percentages were in no case fewer than 500 colonies or cells.

RESULTS

The Yeast *Sch. Pombe* Can Enter a Quiescent State via Many Points in the Cell Cycle . We examined 9 different *Sch. pombe cdc* temperature-sensitive mutant strains for their ability to acquire heat shock resistance following starvation (a measure of entry into a quiescent state) (1, 2). The strains used are (see Table 1): 972 (wild-type); *cdc20* (G_1 arrest); *cdc22* (G_1-S arrest); *cdc17* (S arrest); *cdc23* (S arrest); *cdc24* (S arrest); *cdc25* (G_2 arrest); *cdc2* (arresting at both G_1 and G_2 but mostly at the latter); and *cdc1* (G_2 arrest). Each of these strains was grown in 3 ml of rich YE medium at 25°C for 5 h. During the last 100 min of growth all of the cultures showed a doubling of cell number indicating actively cycling cells. At this point the cultures were shifted to 36°C for 3 h. This resulted in cell cycle arrest for the *cdc* mutants but not the wild-type strain. Microscopic examination of all of the *cdc* mutant strains showed the dramatic elongated cell phenotype (*i.e.*, cell length at least 5 times longer than normal cells) typical of *cdc* mutants of *Sch. pombe* (7, 9). After the 3-h incubation

Received 12/14/92; accepted 2/11/93.

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[1] This work was supported by the Imperial Cancer Research Fund, Medical Research Council (P. N.) and National Cancer Institute Grant CA 50261 and by Grant 2RT0437 from the University of California Tobacco-Related Disease Research Program (D. B.).

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[3] The abbreviations used are: SQ, quiescent state in S phase; *cdc*, cell division cycle; HU, hydroxyurea; BrdUrd, bromodeoxyuridine.

Table 1 *Yeast strains used here*

cdc mutant allele	Species	Arrest point	Source
cdc20-M10	*Sch. pombe*	G_1	P. N. laboratory stock
cdc10-129	*Sch. pombe*	G_1	P. N. laboratory stock
cdc22-MBCII	*Sch. pombe*	G_1-S	P. N. laboratory stock
cdc17-K42	*Sch. pombe*	S	P. N. laboratory stock
cdc23-M36	*Sch. pombe*	S	P. N. laboratory stock
cdc24-M38	*Sch. pombe*	S	P. N. laboratory stock
cdc2-33	*Sch. pombe*	G_1 or G_2	P. N. laboratory stock
cdc25-22	*Sch. pombe*	G_2	P. N. laboratory stock
cdc1-7	*Sch. pombe*	G_2	P. N. laboratory stock
972 (none)	*Sch. pombe*	None	P. N. laboratory stock
cdc25–5	*S. cerevisiae*	G_0	Kelly Tatchell laboratory stock
cdc7–1	*S. cerevisiae*	S	Yeast genetic stock center
cdc8–1	*S. cerevisiae*	S	Yeast genetic stock center
cdc13–1	*S. cerevisiae*	G_2	Yeast genetic stock center
cdc15–2	*S. cerevisiae*	M	Yeast genetic stock center
SP1 (none)	*S. cerevisiae*	None	Michael Wigler laboratory stock

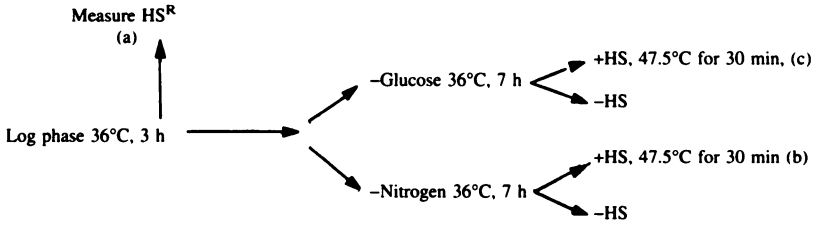
The *Sch. pombe* and *S. cerevisiae cdc* mutant or wild-type strains (972 and SP1) are indicated. The mutant allele of each of the *cdc* mutants is indicated. The arrest point in the cell cycle for the *cdc* mutants is indicated.

at 36°C an aliquot of the cells was removed to determine the percentage of cell resistance to thermal heat shock (47.5°C for 30 min). Serial dilutions of the heat shocked and non-heat shocked cultures were plated onto rich YE medium plates and incubated at 25°C for 3 days. The percentage of heat shock resistance was calculated by dividing the number of colonies appearing after heat shock by the number of colonies appearing after no heat shock treatment. For all experiments described non-heat shocked cultures showed plating efficiencies greater than 65%. The remaining cultures were centrifuged to pellet the yeast cells and washed twice in minimal medium lacking a source of nitrogen or in minimal medium containing only 0.10 g/liter glucose and incubated at 36°C for 7 h, after which aliquots of each culture were assayed for heat shock resistance as described above (see below and Table 2). Prior to starvation conditions all strains examined showed less than 1% survival after the thermal heat shock. In contrast, all strains examined after starvation conditions showed greater than 63% survival after the heat treatments. These results suggest that entry into a quiescent state can occur at many points in the cell cycle.

It is possible that the *cdc* mutants arrested in the cell cycle by shifting to 36°C might have "leaked" past their arrest points and entered quiescence via the normal physiological route (through G_1 for nitrogen starvation and through G_2 for glucose starvation) (4). However, this is unlikely because we had chosen the particular *cdc* mutant alleles because they are known not to have leaky phenotypes. To test this further we also examined the ability of the cells to divide in the presence of 12 mM HU prior to thermal heat shock. HU is a potent inhibitor of DNA replication and causes arrest in S phase (12). Wild-type cells starved of nitrogen arrest in G_1 and cells starved of glucose arrest in G_2 (4). To confirm this point, aliquots of wild-type strain 972 starved for glucose or nitrogen for 7 h at 36°C were diluted and plated on rich YE medium plates containing hydroxyurea and incubated at 25°C for 24 h. Microscopic examination showed that less than 5% of the nitrogen-starved cells and greater than 80% of the glucose-starved cells had undergone cell division on the HU plates, indicating they had indeed arrested in G_1 or G_2 when exposed to the different starvation conditions (see Table 2). Similar controls using HU plates were carried out with diluted aliquots of all of the above-mentioned strains. For example, the *cdc1* mutant, which arrests in G_2 phase, was treated as described above; prior to heat shock aliquots were plated onto rich YE medium plates containing HU. After 24 h at 25°C, >84% of the mutants had undergone a single cell division. If the *cdc1* temperature-sensitive mutant had leaked past its arrest point in the minus-nitrogen starvation condition, we would expect the cells to arrest in G_1 and therefore be unable to divide once on HU plates. Similarly, the *cdc10* mutant, which causes arrest in G_1 phase, was treated as described above; prior to heat shock, aliquots were plated onto YE plates containing HU. After 24 h at 25°C, <5% of the mutants had undergone a single cell division. If the *cdc10* temperature-sensitive mutant had leaked past its arrest point in minus-glucose starvation conditions, we would have expected the cells to arrest in G_2 and therefore be able to divide once on HU plates. A similar test using the hydroxyurea plate assay for leakage past the known arrest point indicated that no significant leakage past any of the *cdc* arrest points we examined had occurred (see Table 2). Thus, by measure of heat shock resistance, *Sch. pombe* cells can enter a quiescent state through G_1, S, or G_2

Table 2 *Heat shock resistance of Sch. pombe arrested mutants after starvation for nitrogen or glucose*

HS, heat shock; HS^R, heat shock resistance. Percentage of HS^R was determined as described in the text at times a, b, and c indicated in the flow chart below. The percentage of cells dividing once in the presence of 12 mM HU was determined by dilution of culture at times b and c and plating cells on YE plates with 12 mM HU and incubating at 25°C for 24 h. The fate of 100 individual cells was microscopically examined for cell division.



		–Nitrogen		Rich medium	–Glucose	
Mutant allele	Arrest point	% dividing on HU (b)	% of HS^R with starvation (b)	% of HS^R without starvation (a)	% dividing on HU (c)	% of HS^R with starvation (c)
cdc20-M10	G_1	3	88	<0.5	6	84
cdc10-129	G_1	4	70	<0.5	4	91
cdc22-MBCII	G_1-S	1	68	<0.5	3	63
cdc17-K42	S	6	75	<1.0	7	69
cdc23-M36	S	8	91	<0.1	7	87
cdc24-M38	S	9	87	<0.5	5	90
cdc2-33	G_2	81	63	<0.5	80	63
cdc25-22	G_2	94	89	<0.1	87	91
cdc1-7	G_2	97	76	<0.5	84	84
Wild-type (972)	(G_1 or G_2)[a]	5	84	<0.1	80	80
	(G_1 or G_2)[a]					

[a] Wild-type *Sch. pombe* cells starved for glucose enter quiescence via G_2, whereas *Sch. pombe* cells starved for nitrogen enter quiescence via G_1. Similar results were obtained in three independent experiments.

phase. It should be noted that heat shock treatment of the *cdc2* mutant strain that had been starved for nutrients resulted in the cells increasing their ploidy. A detailed analysis of this observation has been presented elsewhere (9).

Sch. pombe cdc mutants at the nonpermissive temperature arrest at specific points in the cell cycle yet do not arrest their growth and therefore continue to increase their mass. This gives rise to a typical dramatically elongated phenotype. Furthermore, the continued growth is likely responsible for the loss of viability of *cdc* mutants shifted to 36°C for more than 24 h (2, 13).

We examined the survival of various *cdc* mutants arrested at their known arrest points, either left in rich medium at 36°C for 3 days or shifted to starvation condition for 3 days at 36°C. The starvation conditions used were either minimal medium lacking nitrogen or minimal medium containing only 0.10 g/liter glucose. As seen in Table 3, greater than 63% survival was observed for the *cdc* arrested mutants under starvation conditions, whereas 3 days at 36°C in rich YE medium resulted in a survival rate of less than 3% for all the *cdc* mutants we examined. These results indicate that cells arrested in G_1, S, or G_2 phase of the cell cycle can enter a quiescent state similar to G_0 if exposed to environmental signals which normally induce entry to G_0.

Yeast *S. Cerevisiae* Can Enter a Quiescent State through Many Points on the Cell Cycle. To determine whether entry to a quiescent state via many points in the cell cycle is unique to the yeast *Sch. pombe* or is a more common feature of eukaryotes, we examined the distantly related yeast *S. cerevisiae* for the ability to enter a quiescent state via G_1, S, G_2, and M phases of the cell cycle. For this purpose, we used 5 different *S. cerevisiae* cell division cycle temperature-sensitive mutants: *cdc25–5* (G_0 arrest); *cdc7–1* (S phase arrest); *cdc8–1* (S phase arrest); *cdc13–1* (late G_2 arrest); and *cdc15–2* (M phase arrest), as well as the wild-type strain SP1 (Table 1) (13). To determine whether these *cdc* mutants could enter a quiescent state at their *cdc* arrest point, we followed an experimental procedure similar to that described for the *Sch. pombe cdc* mutants. Each of the *cdc* mutants was grown in rich YPD medium at 25°C to a density equivalent of A_{600} = 0.1 and then shifted to 36°C (the nonpermissive temperature). After 3 h at 36°C, microscopic examination indicated most of the cells had arrested at the *cdc* arrest point. One-half of the culture was used to determine the heat shock resistance of the cells prior to starvation. The other one-half of the culture was pelleted, washed, resuspended in starvation medium (YP medium containing only 0.10 g/liter glucose), and cultured at 36°C for 8 h. To determine heat shock resistance for each of these conditions, cells were either incubated at 50°C for 30 min (heat shock conditions) or left at 36°C, after which serial dilutions were made and plated on rich YPD medium. The plates were incubated at 25°C for 3 days to determine the number of viable cells exposed to the various conditions. Table 4 shows that, without starvation, only the *cdc25-5* mutant showed appreciable resistance to thermal heat shock. This is not surprising since *cdc25* is known to regulate the signal transduction pathway which monitors nutrient availability and hence regulates cell growth (10, 14). Therefore, when a *cdc25* temperature-sensitive strain is shifted to the nonpermissive temperature, it enters G_0; conversely, when a yeast strain harbors a hyperactive *CDC25* allele, it fails to enter G_0 even when starved for nutrients (10, 14). In contrast to the *cdc25–5* strain, all of the other *cdc* mutants examined showed greater than 60% heat shock resistance following starvation conditions. We conclude that the eukaryote, *S. cerevisiae*, can enter a quiescent state via G_1, S, G_2, or M phase of the cell cycle when exposed to starvation conditions.

Table 3 *Long-term survival of Sch. pombe mutants under starvation conditions*

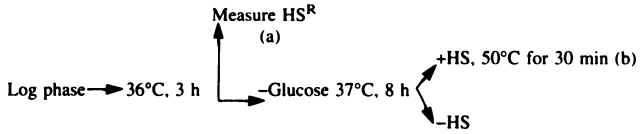
After incubations of the indicated strains for 3 days in rich medium (YE), minus-glucose medium (YE medium with 0.10 g/liter glucose), or minus-nitrogen medium (minimal medium without nitrogen) the number of cells present in serial dilutions was determined by using a Coulter Counter. Aliquots of serial dilutions were plated onto rich medium (YE) plates and incubated at 25°C for 3 days. Percentage of survival was calculated by dividing the number of colonies appearing after 3 days by the number of cells plated.

Mutant allele	Arrest point	% of survival after 3 days at 36°C		
		YE (rich medium)	–Glucose medium	–Nitrogen medium
cdc20	G_1	<1	64	73
cdc22	G_1-S	<1	91	87
cdc17	S	<3	63	84
cdc23	S	<1	66	91
cdc25	G_2	<2	83	81
cdc2	G_2	<1	65	74
cdc1	G_2	<1	75	84
Wild-type	(G_1 or G_2)[a]	92	83	87
	(G_1 or G_2)[a]			

[a] Wild-type *Sch. pombe* cells (strain 972) starved for nitrogen enter quiescence via G_2, whereas *Sch. pombe* cells starved for glucose enter quiescence via G_1. Similar results were obtained in two independent experiments.

Table 4 *Heat shock resistance of S. cerevisiae cdc arrested mutants after starvation for glucose*

Percentage of heat shock resistance (HS^R) was determined as described in the text at times a and b as indicated in the flow chart below, for the indicated *cdc* arrested mutants which cause growth arrest at the indicated points in the cell cycle. Similar results were obtained in three independent experiments.



Mutant allele	Arrest point	% of HS^R with starvation (b)	% of HS^R without starvation (a)
cdc25-5	G_0	100	99
cdc7-1	S	>60	<0.4
cdc8-1	S	>61	<0.2
cdc13	G_2	>71	<2.5
cdc15–2	M	>90	<10
Wild-type (SP1)	G_1-G_0 (–glucose)	>60	<0.1

DISCUSSION

Eukaryotic cells which have not been exposed to environmental signals stimulating the mitotic cell cycle enter a quiescent state, leaving the cell cycle. For most eukaryotic cell types it is generally thought that the entry into quiescence occurs via G_1, into a state often referred to as G_0. However, some eukaryotic cell types can enter a quiescent state via points in the cell cycle other than G_1. The yeast *Sch. pombe* enters a quiescent state through G_2 when starved for glucose (4). In *Pisum*, the root meristem contains cells which can enter quiescence through G_1 or G_2 (15). In the ciliated protozoan, *Tetrahymena pyriformis*, entry into quiescence can occur via G_1 or G_2 depending upon cell density at the time of nutrient depletion (16). A reversible arrested state in G_2 has also been observed for mammalian epidermal cells (17, 18) and murine B lymphocytes (19).

Thus, it appears that eukaryotes do possess mechanisms which can operate in G_1 or G_2 to allow cells to leave the cell cycle and enter a quiescent state in which they remain viable for extended periods and from which they can reenter the cell cycle. The results presented here indicate that entry to that stable-quiescent state for the distantly related yeasts, *Sch. pombe* and *S. cerevisiae*, can occur at all points in the cell cycle examined here (including points in G_1, S, G_2, or M) when the cells are exposed to conditions which normally signal entry to G_0. Furthermore, these results make clear the distinction between cell cycle arrest and growth arrest. Yeast *cdc* mutants arrest at specific points in the cell cycle but only arrest growth and enter a stable-quiescent state when exposed to starvation conditions. These experiments made use of cell division cycle mutants for which the vast majority of the cells in the culture could be arrested at a specific point in the cell cycle so that large numbers of cells could be examined for

entry into a quiescent state. Thus it appears that the mechanisms which regulate exit from the cell cycle and entry into a quiescent state can function in G_1, S, G_2, and M phase and are not limited to cells in G_1 (or G_2). Furthermore, the mechanisms which regulate exit from the quiescent state and reentry into the cell cycle can also function in G_1, S, G_2, and M phases.

The presence of SQ cells has been observed in mammalian cells treated with differentiation-inducing agents such as retinoic acid, *n*-butyrate or dimethyl sulfoxide (5). Because the measurement used to detect the SQ cells made use of indirect methods, *i.e.*, flow cytometry, the existence of an SQ state is not generally acknowledged. Our observation that entry into a SQ-like state for *Sch. pombe* and *S. cerevisiae* suggests that the mechanism by which eukaryotic cells enter a quiescent state can occur at any point in the cell cycle and thus supports the existence of an SQ state.

Analysis of human solid tumors by flow cytometry has consistently shown a discrepancy between the number of cells in S phase as detected by DNA content and the number of cells in S phase as monitored by incorporation of BrdUrd (20, 21). Consistently, a higher percentage of S phase cells as measured by DNA content relative to the percentage measured by BrdUrd incorporation has been observed. One possible explanation for this discrepancy is that some cells have arrested in S phase and entered a quiescent state and have ceased replicating DNA, and so would not take up BrdUrd. Such tumor cells would likely be resistant to chemotherapeutic agents which cause lethal defects in DNA synthesis. The results presented here and by others (5) are consistent with the existence of quiescent S phase cells. Consequently, different strategies may be required to selectively kill tumor cells that re enter the cell cycle from G_0 *versus* SQ.

ACKNOWLEDGMENTS

We are grateful to John Nitiss and Ambrose Jong for providing *S. cerevisiae* cell division cycle mutants. We thank Zbigniew Darzynkiewicz, Richard Moran, and Shirley Taylor for advice and discussions.

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Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Cancer Res 1993;53:1867-1870.