High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by *trans*-complementation of *Schizosaccharomyces pombe*

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ABSTRACT

We describe a highly efficient alkali cation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of fission yeast *Schizosaccharomyces pombe* mutants. cDNA libraries constructed with the pcD or pcD2 vector are transduced into yeast by cotransfection with a linearized vector, which allows an enhanced homologous recombination between the yeast vector and the library plasmid leading to the efficient formation of concatemers containing pcD molecules. The transformation frequencies obtained by the method are 10^6 colonies per 10^8 cells transfected with 2 μg of library and 1 μg of vector, 50–60% of which contain pcD molecules. The high-efficiency alkali cation method circumvents many of the shortcomings of the spheroplast method generally used for *Schiz. pombe* transfection. The vectors are maximized for the efficiency of library transduction and minimized for the rearrangements of pcD molecules during propagation in yeast.

This system allows rapid screening of multi-million cDNA clone libraries for rare cDNAs in a routine scale of experiments. Using this system, various mammalian cDNAs that are extremely difficult, time-consuming, or unclonable to clone by other methods have been cloned.

INTRODUCTION

Despite the past remarkable success (1–3), isolation of genes by complementation of mammalian cells is in general extremely laborious, difficult and many times impossible because of the requirement of special facilities, slow growth rates, frequent phenotypic reversions and a lack of appropriate mutant cells.

The fission yeast *Schizosaccharomyces pombe* resembles to higher eukaryotes in the structure and function of genes and encoded proteins. Unlike *Saccharomyces cerevisiae*, *Schiz. pombe* recognizes various mammalian promoters including the SV40 promoter (4–5), splices mammalian introns (6), and shares the same polyadenylation signals with mammalian cells. These facts indicate that *Schiz. pombe* is evolutionally related to higher eukaryotes so closely that many *Schiz. pombe* genes might be functionally substituted by mammalian counterparts. In fact, Lee and Nurse (7) have recently isolated the human homolog cDNA of the *Schiz. pombe* cell cycle control gene *cdc2*$^+$ by direct complementation of a yeast mutant with a human cDNA library constructed with pcD, a cDNA cloning vector in which the SV40-derived DNA segments were arrayed to permit transcription, splicing, and polyadenylation of inserted cDNAs in mammalian cells (8). Cloning of mammalian genes based on complementation of *Schiz. pombe* mutants has thus been demonstrated to be possible. However, the poor transfection frequencies ($2-3\times10^4$/μg)(9), the interference of selection for complemented cells by sorbitol that is used for the regeneration of glucanase-digested cell walls in the spheroplast method, and a tendency of frequent rearrangements of the transduced pcD plasmid (7) have been major obstacles to fully pursue this approach. We have circumvented these problems by devising a highly efficient alkali cation method and yeast vectors maximized for the frequencies of library transduction and the stability of transduced pcD molecules.

EXPERIMENTAL PROCEDURES

Yeast strains

The *Schiz. pombe* strains [genotype: *ura4–294 h*$^-$ (ATCC38436) and *leu1–32 h*$^-$ (ATCC38399)] were used as the standard hosts for transformation assay.

Media and reagent

The composition of MB medium was the same as that of MML (10) except that KH_2PO_4 was reduced from 0.1% to 0.05%, glucose from 1% to 0.5% and asparagine replaced with 0.036% potassium acetate. All solutions and media used for yeast transformation were prepared with mili-Q (Milipore) water and sterilized with disposable nitrocellulose membrane filter units

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(pore size 0.45 μm). Polyethylene glycol-4000 was purchased from Fluka, and Bacto-yeast extract from Difco.

Transformation by the high-efficiency alkali cation method

Yeast cells were grown on a YEA plate (10) for 1 or 2 day(s). A loop of combined yeast colonies was inoculated into 150 ml of MB medium supplemented with 150 μg/ml of uracil (for *ura4*$^-$) or leucine (for *leu1*$^-$) in a 2 l-flask and grown to a density of $0.5-1\times10^7$ cells/ml with moderate shaking at 32°C. Cells were collected by centrifugation at $1,000\times g$ for 5 min, and washed twice with water and suspended in an appropriate volume of 0.1 M lithium acetate (adjusted to pH of 4.9 with acetic acid) to make the cell concentration 1×10^9/ml. The cell suspension was dispensed by 0.1 ml aliquots into Eppendorf tubes and incubated for 60 min in a water bath at 30°C. Fifteen μl of DNA solution in 10 mM Tris-hydrochloride (pH 7.5) buffer containing 1 mM EDTA was added to each tube. The tube was then vortexed gently and incubated for 60 min at 30°C. This 60 min incubation is not required for at least some strains. After gentle vortexing to resuspend cells that had sedimented during the incubation, 290 μl of 50% (w/v, in water) polyethylene glycol-4000 (preincubated at the same temperature) was added and mixed again by gentle vortexing. Incubation was continued for another 60 min at 30°C. The cells were then heat shocked by placing the tubes in a water bath at 43°C for 15 min and, after cooling down at room temperature, pelleted by centrifugation at $5,000\times g$ for 2 min. Pelleted cells were suspended in 1 ml of 0.25% Bacto-yeast extract containing 1.5% glucose and 30 μg/ml of uracil (for *ura4*$^-$) or leucine (for *leu1*$^-$) and inoculated into a 200 ml-flask containing 9 ml of the same medium. The flask was incubated at 32°C for 30–120 min with vigorous shaking. Fractions of the cell suspension were then spread on MMA plates (10) and incubated for 4–7 days. For temperature sensitive mutants, all incubation described above was done at the permissive temperature except for the step of heat shock, and the last shaking was continued for 2–4 hours.

RESULTS AND DISCUSSION

High-efficiency alkali cation method

The major factors that have contributed to the high efficiency of our method are: 1) culturing recipient cells in the modified minimal medium (MB medium), relatively poor medium as opposed to rich medium (11–12), 2) treating cells with lithium acetate at a low pH, and 3) optimization of all the steps of transfection.

We initially observed significantly higher transformation frequencies by replacing the medium in the original alkali cation method (YEL or YPD) with minimal medium (MML) for culturing the recipient cells. Subsequently we found that asparagine included in MML had adverse effects on the transfection frequency. Therefore, it was replaced by potassium acetate as a pH stabilizer. Reduction of the glucose concentration in MML from 1% to 0.5% improved frequencies 2–3 fold. Growth in rich medium (YEL or YPD) always yielded low frequencies (less than 10^4/μg).

Cells that have been grown in MB medium seem to have a much thinner cell wall. They were fragile but highly viable (>80%). This seems to be a reason for the remarkable increase in the effectiveness of the alkali cation treatment on the cells. In fact, cells that have been grown in this medium have already

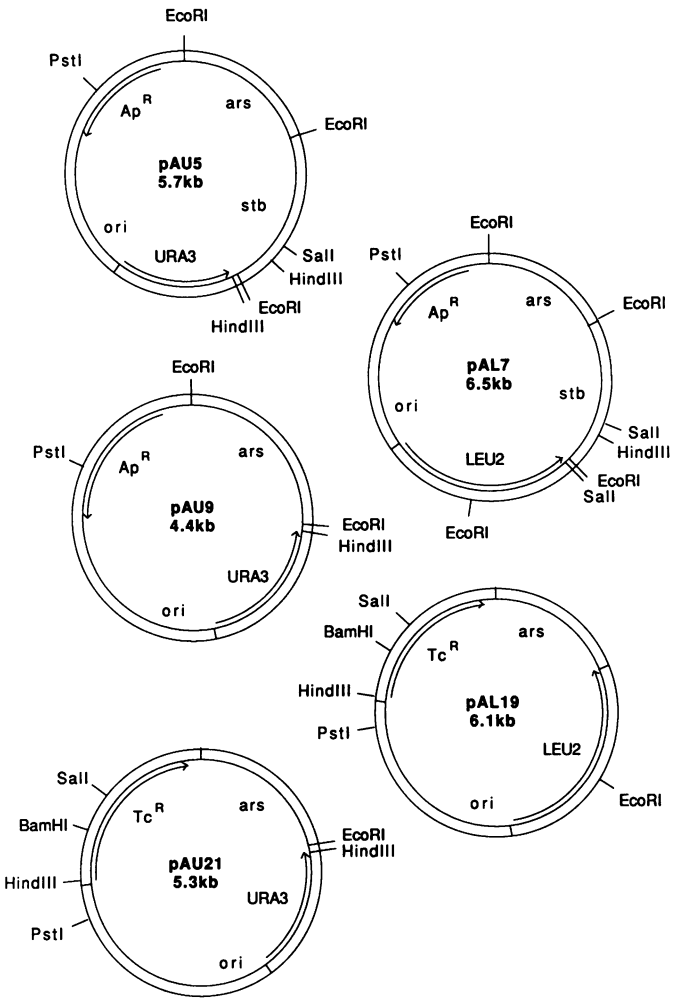

Figure 1. The structure of the transducing vectors. In these vectors, the end of pBR sequence created by *Pvu*II digestion was joined to the *URA3* coding sequence released by *Pst*I digestion and subsequently blunt-ended (the pAU series), or to the *LEU2* coding sequence released by *Hinc*II digestion (the pAL series). These vectors share the pBR sequence spanning from the *Pvu*II site to the *Eco*RI site (pAU5, pAL7, and pAU9) or to the *Sca*I site (pAL19 and pAU21) with pcD.

Table 1. Transformation efficiencies of transducing vectors

Host	Plasmid[a]	Transformants $\pm\sigma_n(\times10^{-5})/\mu g$[b]
ura4–294 h$^-$	pFL20	4.6 ± 0.9
(n=2)[c]	pAU5	7.2 ±0.5
	pAU9	11.1 ± 0.1
leu1–32 h$^-$	pDB262	2.8 ± 0.3
(n=2)	pAL7	6.9 ± 0.9
	pEL11	5.5 ± 1.0
ura4–294 h$^+$	pFL20	4.2
(n=1)	pAU5	6.3
	pAU9	9.1
pat1–114 ura4–294 h$^-$	pAU5 (n=5)	2.7 ± 0.3
	pAU9 (n=2)	8.3 ± 1.0

[a] The size of plasmids is: pFL20, 8.0 kb; pAU5, 5.7 kb; pAU9, 4.4 kb; pDB262, 10.6 kb; pAL7, 6.5 kb; pEL11, 7.7 kb.
[b] One μg of circular DNA was used for transfection to determine transformation frequencies.
[c] The number of independent preparation of competent cells. Each score was the average of results in duplicated experiments.

been made competent to some extent by the action of the potassium ion and the low pH in the medium during growth. Without the lithium acetate treatment, they were transformed at an frequency of $7\times10^3/\mu g$.

Consistent with the previous report on *Sacch. cerevisiae* transfection (11), lithium was the most effective among the alkali cations tested (Li, Na, K, Rb, and Cs), and acetate better than chloride (data not shown). Remarkably, the efficiency sharply depended on the pH of lithium acetate (Fig. 2). The optimal pH ranged from pH 4.9 to pH 5.1. The viability of the cells at this pH range was slightly lower. The same pH dependency was also observed when potassium acetate or sodium acetate was used (data not shown).

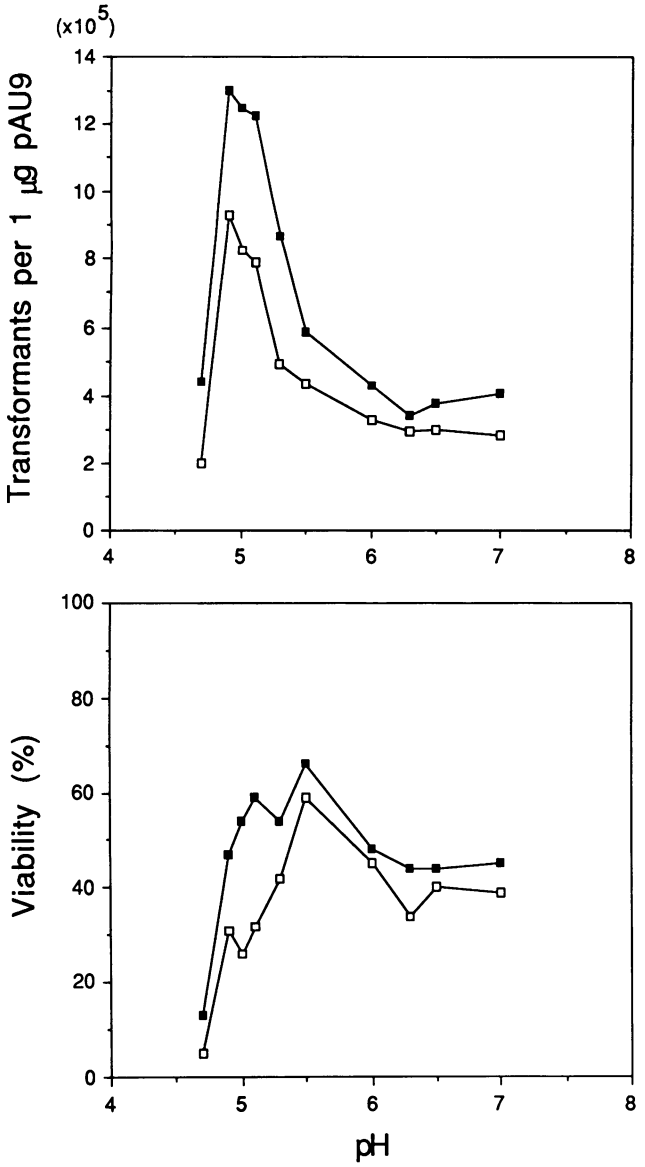

Figure 2. Transformation frequencies as a function of the pH of the lithium acetate solution. The *Schizosaccharomyces pombe* strain (genotype, *ura4−294 h*$^-$) was transfected with 1 μg of pAU9 as described in section a except that the lithium acetate solution with various pH adjusted with acetic acid was used. Upper figure shows the number of ura$^+$ transformants/μg pAU9 per 10^8 cells. Lower figure is the cell viability after transfection as indicated by the percentage of the cells that formed colonies on YEA plates after transformation. The results are from two independent experiments (■ and □).

Recently, Allshire (13) reported an improved spheroplast method to transform *Schiz. pombe* using a liposome-forming reagent at the nearly same frequency ($7\times10^5/\mu g$) as ours. In this method, however, the cell wall has to be regenerated in a medium containing sorbitol, which often interferes with the subsequent selection of colonies transduced with a library.

Transducing vectors

We next optimized yeast vectors for the expression of the auxotrophic marker genes and the stability of concatemers made of the yeast vector and the library (Fig. 1 and legend). The parent plasmids are pFL20 (14) and pDB262 (15). The transducing vectors constructed, pAU5, pAU9, pAU21, pAL7, pAL19, have 1) a *Schiz. pombe* autonomously replicating sequence (*ars*)(14,16), and 2) the leucine or uracil auxotrophic selection marker gene transcribed by a strong promoter residing in the pBR322 sequence near *ori*, and 3) the pBR322 sequence containing Ap^R or Tc^R gene and *ori*. pAL19 and pAU21 have the Tc^R marker instead of Ap^R to achieve the preferential recovery of the pcD plasmid in *Escherichia coli* (Fig. 1). These two plasmids are as efficient for transformation as pAU9 and are especially suitable for the recovery of pcD which is present in yeast colonies only at a low copy number. Unnecessary sequences were trimmed off to minimize the size of the vectors. Consequently these vectors are several fold more efficient for transformation than the parents (pAU5 or pAU9 vs pFL20 for *URA3*, and pAL7 vs pDB262 for *LEU2*)(Table 1) and stable when concatemerized with the pcD library. pEL11, which was identical to pAL7 except that the *ars* and *stb* sequences were replaced with the 2μ sequence, transformed cells as efficiently as pAL7. However, colonies obtained with pEL11 were extremely heterogeneous in size, and tended to induce a drastic rearrangement in the cotransfected pcD plasmid during amplification in yeast as seen with the other 2μ-based vectors (data not shown; ref. 16). The *stb* element (16) had no effect on either the transformation frequency (pAU5 vs pAU9) or the stability of transduced pcD plasmids. On the other hand, the genetic background of host cells affected transformation frequencies, perhaps as a reflection of lowered viability due to increased sensitivity to various treatments during transfection or as a result of unfavorable transfection conditions (low temperature for temperature sensitive mutants)(Table 1 and data not shown). The mating type did not affect the frequency.

Transduction and recovery of cDNA library

A cDNA library in the pcD vector, being unable to replicate by itself in *Schiz. pombe*, is transduced into the yeast by cotransfection with a yeast vector, which results in the formation, by homologous recombination, of concatemers that are maintained as an episomal DNA (17). We have found that use of the vector linearized with *Pst*I markedly increase the number of the total transformants as well as the frequency of cotransformation. pcDura, the pcD vector containing the *URA3* coding region, was transfected into a *Schiz. pombe leu1−32 ura4−294* mutant together with various amounts of circular or *Pst*I-linearized pAL7 (Fig. 3). At any amount, transfection with the linearized vector yielded a significantly higher number of both double and leu$^+$ single transformants. The frequency of cotransformation doubled to 50−60%. This effect was not seen with the spheroplast method, perhaps because competent cell suspension prepared by the spheroplast method was full of nucleases which degraded linear DNA.

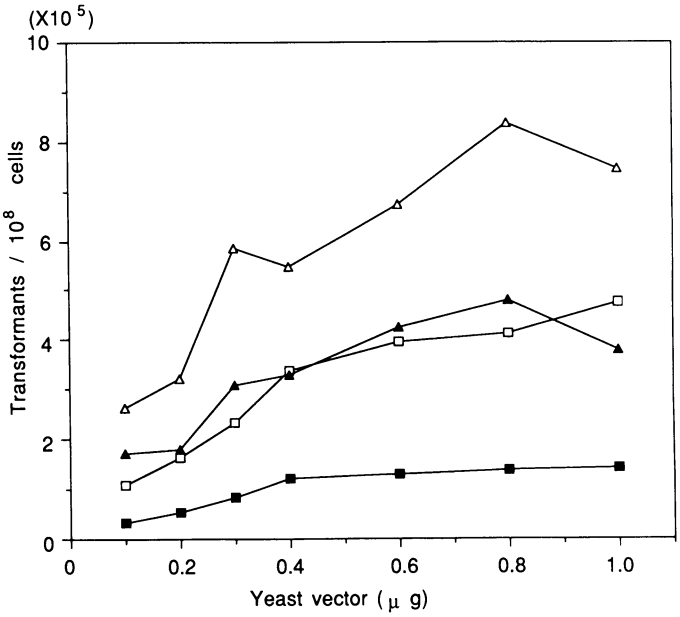

Figure 3. Cotransformation with pcDura and circular or linear pAL7. The *Schizosaccharomyces pombe* strain (genotype, *ura4−294 leu1−32 h⁻*) was transfected with a mixture of pcDura (2 μg) and various amounts of circular or *Pst*I digested pAL7 (0.1−1 μg) as described in Experimental procedures. Transfected cells were spread on plane MMA plates or MMA plates containing 50 μg/ml of uracil. leu$^+$ colonies were selected on uracil-containing MMA plates after transfection with circular (□) or linear (△) pAL7, and leu$^+$, ura$^+$ doubly transformed colonies selected on MMA plates after transfection with circular (■) or linear (▲) pAL7. The transformation efficiency of this competent cell preparation was 8.5×10^5/μg when 1 μg of circular pAL7 was used.

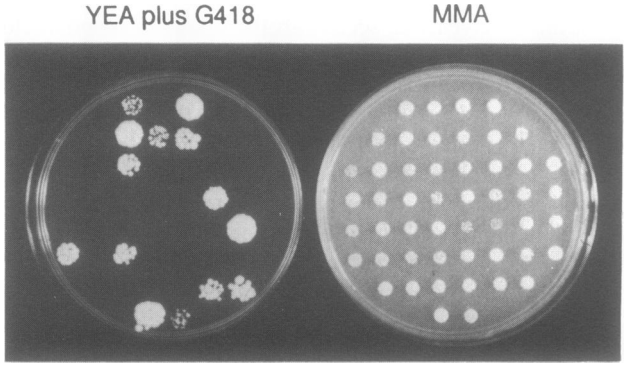

Figure 4. G418 resistance test for identification of colonies containing pcD2 plasmid. The temperature sensitive mutant (genotype, *pat1−114 ura4−294 h⁻*) was transfected with the pcD2-Basinger human primary fibroblast cDNA library together with *Pst*I-digested pAU9. Colonies were selected on minimal plates at the restrictive temperature (34°C), isolated and maintained for three cycles of colony formation on minimal plates at 34°C. Each colony (about 1 mm diameter) from the last plate was suspended in 3 ml water. Three μl of the suspension was spotted on a YEA plate containing 25 μg of G418 /ml (as 100% potency) or on a MMA plate, and incubated at 34°C for 5 days. Left, YEA plate containing G418; right, MMA plate.

The G418^R marker in the pcD2 vector (18) is used for distinguishing cotransformants from transformants carrying only the vector plasmid (Fig. 4). When 2 μg of pcD2 library was transfected into a *ura4⁻* strain together with 1 μg of *Pst*I-linearized pAU9, about 1×10^6 ura$^+$ transformants were obtained, and a half of them were G418^R. This G418^R selection

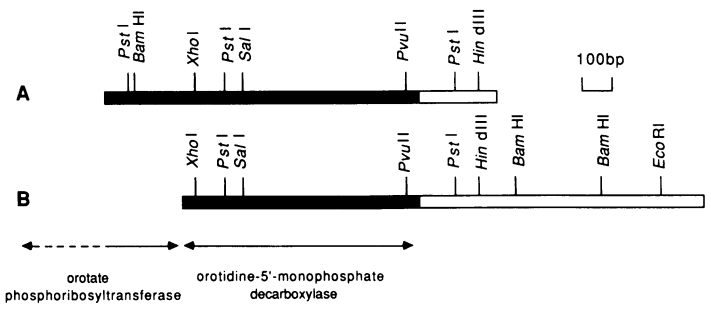

Figure 5. Restriction map of a mouse orotidine-5′-monophosphate decarboxylase cDNA isolated by complementation of a *Schizosaccharomyces pombe ura4⁻* strain. A, a mouse cDNA isolated by complementation of *ura4⁻* mutant; B, a mouse cDNA encoding the orotidine-5′-monophosphate decarboxylase domain of uridylate synthase (23). Filled bars indicate the protein coding region. Upon transfection of *ura4⁻* mutant with a mouse fibroblast library (a total 9×10^5 leu$^+$ colonies), one colony grew on MMA plate, from which one cDNA clone was recovered. The cDNA had a shorter 3′ non-coding sequence but a longer 5′ sequence that encodes part of the orotate phosphoribosyltransferase domain which constitutes one large polypeptide chain together with orotidine-5′-monophosphate decarboxylase whereas they are encoded by separate genes in yeast.

is particularly useful when trans-complementation cloning has to be done with mutant cells that tend to phenotypically revert at a high frequency. A typical result of the G418 plate test is shown in Fig. 4. The temperature sensitive *pat1−114* mutant (19) produced a high number of background colonies derived from epigenetic reversion. During passages of colonies, the concatemers of the pcD clone and the yeast vector are often segregated by an intramolecular homologous recombination, and the pcD2 plasmid is lost from the yeast cells if no selective pressure is placed for the presence of the cDNA. Consequently, phenotypic revertants are often sensitive to G418.

To recover cDNA clones, small molecular weight DNA was prepared from yeast colonies by the Hirt method (20) and transfected into highly competent RecA$^-$ *E. coli* HB101 (21). Recovered plasmids were mostly concatemers and sometimes monomers of either the transducing vector or the pcD plasmid, and few found to have undergone rearrangements when these strains were used. Use of RecBC$^-$ strain BJ5183 as a recipient often resulted in the recovery of plasmids extensively rearranged by deletion and duplication.

When the recovered pcD2 plasmids are reintroduced into yeast cells to confirm the complementation activity, the transformation can be scaled down to 1/10 and plasmid DNA prepared by the small scale rapid alkaline lysis method (22) can be used without a significant reduction of the efficiency.

Cloning of mammalian cDNA by trans-complementation of fission yeast

In order to test the efficiency and power of this system, we screened a mouse fibroblast cDNA library for a orotidine-5′-monophosphate decarboxylase cDNA since the *ura4⁻* mutant could be used as a host for cloning. We isolated within 3 weeks such a cDNA which overlaps the cDNA cloned by Ohmstede et al. (23) but is significantly extended to the 5′-direction and polyadenylated at a different site (Fig. 5). We have also isolated human homologs of *cdc25*$^+$ and *wee1*$^+$, *Schiz. pombe* cell cycle control genes (Nagata et al., Igarashi et al. in preparation).

One of the advantages of expression cloning is that it allows isolation of the gene of the mutation as well as some other genes

that phenotypically complement or suppress the mutant used as a host. This property of expression cloning is often extremely useful for isolating genes that directly or indirectly interact with the mutated gene. They could be genes that share the function with the mutated gene, genes that work downstream of the mutated gene, genes whose protein products form a complex with the mutant protein, thereby stabilizing it, or genes that induce overexpression of the mutated gene. In fact, we have cloned several human cDNAs that suppress *Schiz. pombe pat1*$^-$, a differentiation-proficient temperature sensitive mutant (Okazaki et al. unpublished results) and a human cDNA that is not a *cdc2*$^+$ homolog but suppresses the temperature-sensitive *cdc2*$^-$ mutation (Benton et al. unpublished results).

There are available a large collection of *Schiz. pombe* mutants that appear to serve as excellent hosts for cloning mammalian counterparts (24). Among them are those involved in cell cycle control (25–27), meiotic recombination (28), DNA repair (29–30), and pre-mRNA splicing (31). In addition, mutants for various cellular and nutritional metabolisms exist already.

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