Large-scale screening of intracellular protein localization in living fission yeast cells by the use of a GFP-fusion genomic DNA library

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Abstract

***Background*: Intracellular localization is an important part of the characterization of a gene product. In an attempt to search for genes based on the intracellular localization of their products, we constructed a green fluorescent protein (GFP)-fusion genomic DNA library of *S. pombe*.**

***Results*: We constructed the *S. pombe* GFP-fusion genomic DNA library by fusing, in all three reading frames, random fragments of genomic DNA to the 5′ end of the GFP gene in such a way that expression of potential GFP-fusion proteins would be under the control of the own promoters contained in the genomic DNA fragments. Fission yeast cells were transformed with this plasmid library, and microscopic screening of 49 845 transformants yielded 6954 transformants which exhibited GFP fluorescence, of which 728 transformants showed fluorescence localized to distinct intracellular structures such as the nucleus, the nuclear membrane, and cytoskeletal structures. Plasmids were isolated from 516 of these transformants, and a determination of their DNA sequences identified 250 independent genes. The intracellular localizations of the 250 GFP-fusion constructs was categorized as an image database; using this database, DNA sequences can be searched for based on the localizations of their products.**

***Conclusions*: A number of new intracellular structural components were found in this library. The library of GFP-fusion constructs also provides useful fluorescent markers for various intracellular structures and cellular activities, which can be readily used for microscopic observation in living cells.**

Introduction

The intracellular localization of its product can offer important clues as to the function of an unknown gene. Recently, there have been several reports on the construction and screening of DNA libraries designed to allow an intracellular visualization of expressed gene products: the genome of the budding yeast *Saccharomyces cerevisiae* was screened using LacZ-fusion constructs, and a number of genes were successfully categorized based on their localization patterns (Burns *et al.* 1994); in the fission yeast *Schizosaccharomyces pombe* a GFP-fusion genomic library that had been constructed utilizing the inducible nmt1 promoter, was first screened for DNA fragments which affected mitotic cell growth when expressed from the nmt1 promoter. These selected DNA fragments were then screened for the intracellular localization of their products (Sawin & Nurse 1996); and more recently, a GFP-fusion human cDNA library was constructed, and a microscopic screening of the library identified a novel nuclear envelope protein (Rolls *et al.* 1999).

Here we report the construction of a green fluorescent protein (GFP)-fusion genomic DNA library of *S. pombe* in which the authentic promoters of the genes which are fused to GFP regulate the expression of the fusion constructs. This strategy allowed us to examine the intracellular localization of gene products expressed under physiologically relevant conditions. Based on our screening of this library, we selected 516 transformants with GFP staining in distinct intracellular structures, such as the nucleus, the nuclear membrane, spindle-pole body (SPB), microtubules, and other

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cytoskeletal/cytoplasmic structures. A determination of the DNA sequences of the plasmids isolated from these transformants identified 250 independent genes, which we categorized based on the intracellular localization of their GFP-fusion products. Using this image database, it is possible to search for gene products which compose intracellular structures of interest. One notable advantage of the use of GFP-fusion is that the cloned DNA is ready for use in living cells. Our library of GFP-fusion constructs also provides useful fluorescent markers for various intracellular structures and cellular activities.

Results

Microscopic screening of a GFP-fusion genomic DNA library

Our strategy for the construction and screening of the GFP-fusion genomic DNA library is shown in Fig. 1. Using a multicopy plasmid vector containing no foreign promoters, we constructed three plasmids with GFP gene inserts, each plasmid designed to accept a genomic DNA fragment at the 5′ end of the GFP gene such that the GFP gene is in one of three reading frames in relation to the DNA fragment (see Experimental procedures); thus, only those recombinant plasmids in which the genomic DNA insert contains a gene together with its own promoter, and in which the gene is fused in-frame with the GFP gene are able to express a fluorescent protein.

S. pombe cells of a homothalic h^{90} strain, CRL126, were transformed with the GFP-fusion genomic DNA libraries, and transformed cells were grown individually on EMM2 plates (see Experimental procedures). Under these conditions, a relatively small proportion of the cell population enters meiosis, while the majority grow mitotically. Because the genes fused with GFP were expressed under their own promoters, it was possible to examine the intracellular localization of the gene products under physiologically relevant conditions in a mixture of mitotic and meiotic cells during screening. Meiotic prophase nuclei in the fission yeast can be easily distinguished by their characteristic elongated morphology, generally called 'horse-tail' nuclei (indicated by an asterisk in Fig. 2); the horse-tail nucleus shows an oscillatory movement between the cell poles during meiotic prophase (Chikashige *et al.* 1994; see Fig. 4B).

Of the 49 845 transformants screened, 6954 transformants exhibited GFP fluorescence. Most of the fluorescent transformants gave a uniform GFP staining within the cell and were excluded from further screening (see the legend to Fig. 1), but 728 transformants showed

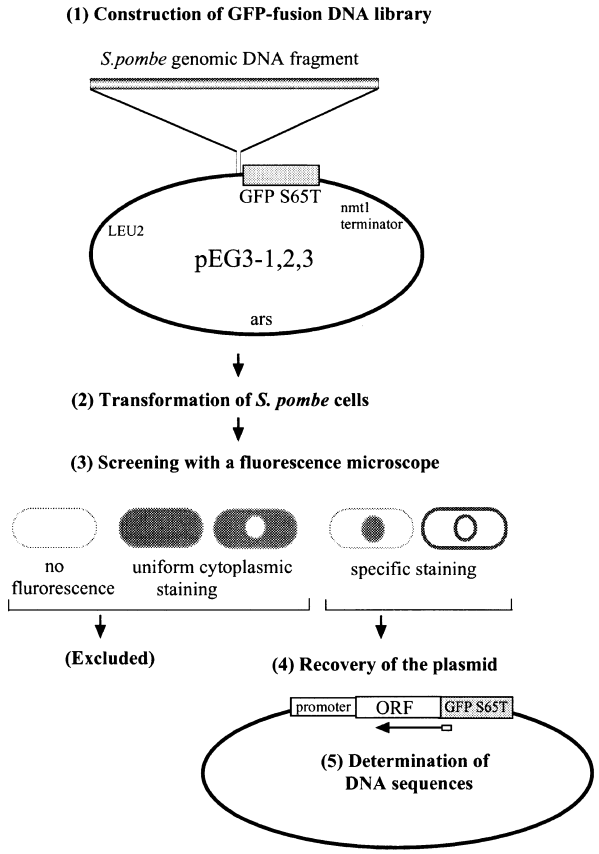

Figure 1 Strategy for construction and screening of the GFP-fusion genomic DNA library. The figure illustrates the processes of: (1) construction of the GFP-fusion library, (2) transformation of *S. pombe* cells with the library, (3) microscopic screening of the *S. pombe* transformants, (4) recovery of plasmids from the selected transformants, and (5) determination of nucleotide sequences of a DNA insert. During microscopic screening, we excluded those transformants which showed no fluorescence, uniform staining throughout the cytoplasm and the nucleus, and uniform staining in the cytoplasm with nuclear exclusion.

fluorescent staining in distinct intracellular structures (Table 1). Plasmids were recovered from 516 of these latter transformants and partial sequences of the inserted DNA were determined. The 516 cloned DNA sequences identified 250 independent genes. The sequenced plasmids were retransformed to a different h^{90} strain, CRL152, to confirm that the localization patterns were reproducible.

We categorized the 250 gene products into 11 groups based on their intracellular localizations (Table 1): nuclear components (subdivided into the general nuclear region, the nucleolus, nuclear dots and the nuclear rim), general membrane components, SPB components, microtubule components, cell wall components, septum and cell

pole components, other cytoskeletal/cytoplasmic components, and spore wall components. We examined both mitotic and meiotic cells; only three of the 250 genes were found to be expressed specifically in meiotic cells. In one of these three transformants, TA27, the GFP signal can be detected in nuclei only from meiotic prophase to the first meiotic division, and the other two transformants, A799 and TB91, exhibited brightly stained spore rims (Table 2; image data not shown).

DNA sequences determined for these 250 constructs were compared with public databases of DNA sequences; the results are summarized in Table 2. The determined DNA sequences of the 250 gene fragments were submitted to the public database DDBJ with comments on intracellular localization of their products. Accession numbers are AB027768–AB028018 (Table 2; although DNA sequences of 251 gene fragments were submitted, AB027963 turned out to be identical to AB027870).

Construction of the image database of intracellular localization

We constructed an image database of the intracellular localization of these gene products categorized into the 11 groups; using this database, DNA sequences can be searched based on the localization of their products. Occasionally, a GFP-fusion protein was observed in more than one intracellular localization. In these cases, we categorized the gene by the intracellular location which exhibited the most prominent staining. Secondary localizations are also listed in the database, and genes can be searched for by any of their detected locations.

(i) Nuclear components

The nuclear component category, containing 145 of the 250 genes cloned in this study, is subdivided into four groups (Table 1): 98 genes for general nuclear staining (Fig. 2A), 10 genes for nuclear dots (Fig. 2B), 22 genes for nucleolar staining (Fig. 2C), and 15 genes for nuclear rim staining (Fig. 2D). The search for a nuclear localization signal (NLS) in these constructs using the PSORT II program <http://psort.ims.u-tokyo.ac.jp> (Nakai & Horton 1999) found putative NLSs in 64 constructs (for details, see Table 3).

Examples of general nuclear staining are shown in Fig. 2A for A691, M730 and TC35. A691 is an unknown gene; TC35 is an unknown gene containing a myb domain; and M730 is a homologue of *S. cerevisiae* RNA helicase PRP43 (Table 2). General nuclear staining includes staining of the chromatin region (TC35 in Fig. 2A), as well as staining of the entire nuclear region. In some cases, as shown in A691 (Fig. 2A), variable cytoplasmic staining was also observed. Putative NLSs

Table 1 Summary of GFP-fusion library

	Frame 1	Frame 2	Frame 3	Total
Total clones screened	25 494	12 084	12 267	49 845
Clones with GFP staining	3295	1279	2380	6954
Clones with discrete GFP staining patterns	302	130	296	728

Localization pattern	Total number	Sequenced clone	Independent gene
Nucleus (general nuclear staining)	307	236	98
Nuclear dots	31	26	10
Nucleolus	29	29	22
Nuclear rim	26	26	15
Membrane (nuclear and plasma membrane, ER)	180	115	57
SPB	6	6	3
Microtubule	22	22	5
Cell pole, septum	10	12	8
Cell periphery	26	14	11
Cytoplasmic structures	85	25	19
Spore rim	6	5	2
Total	728	516	250

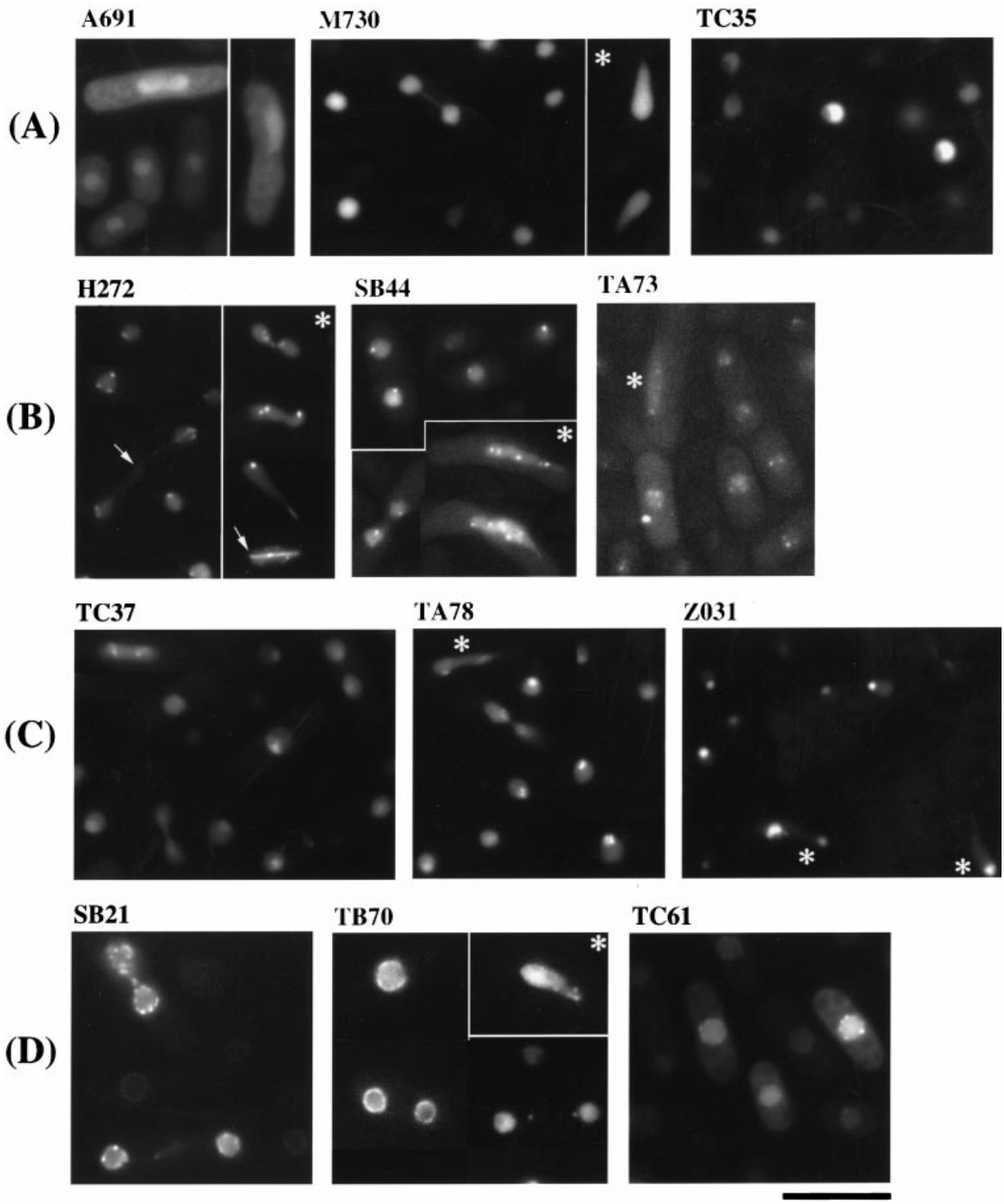

Figure 2 Nuclear components. (A) general nuclear staining, (B) nuclear dots, (C) nucleolus, and (D) nuclear rim. Asterisks indicate conjugated zygotes, in which fusing nuclei during karyogamy and elongated, 'horse-tail' nuclei in meiotic prophase can be seen. Scale bar represents 10 μm.

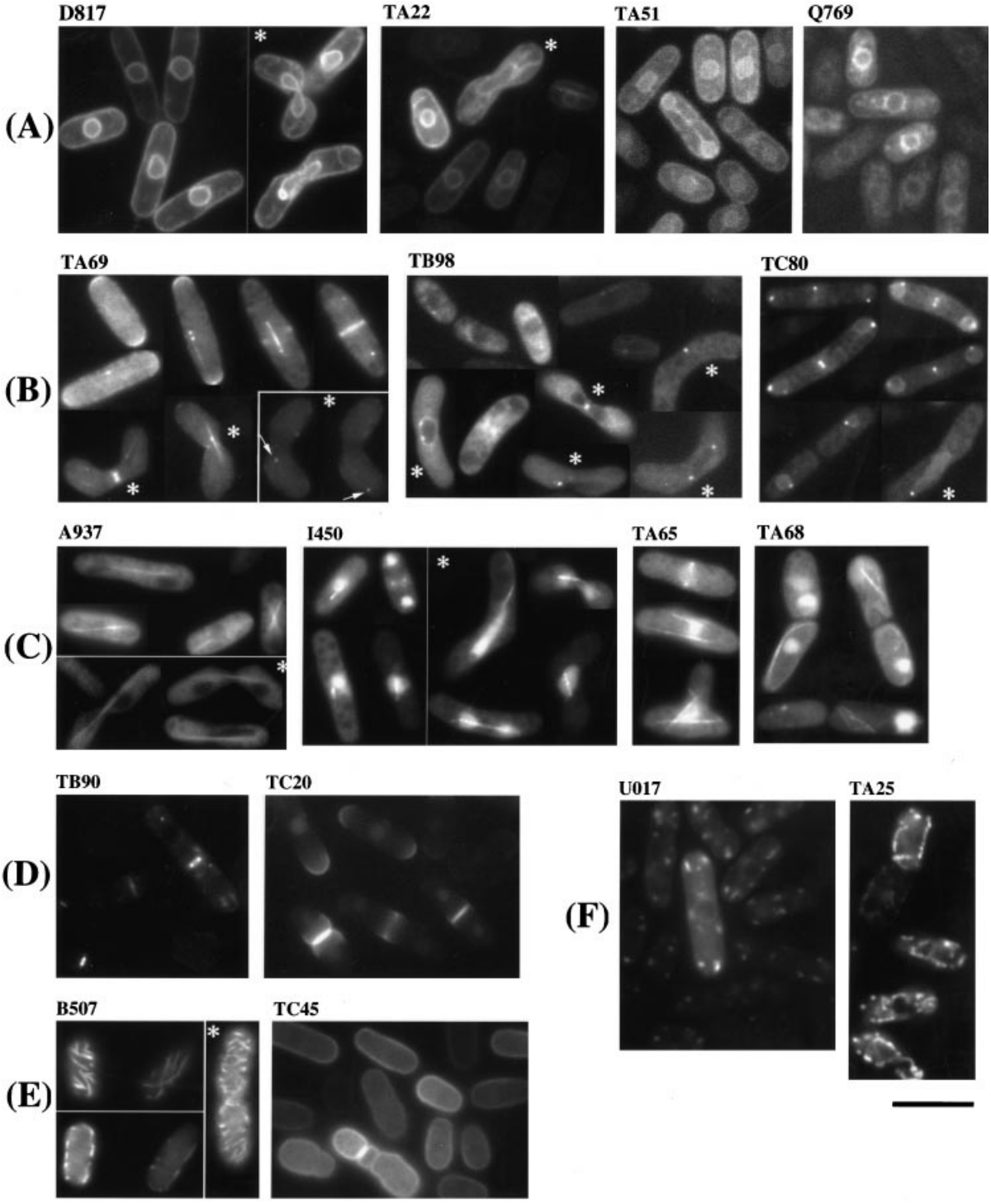

Figure 3 Membrane, cytoskeletal and cytoplasmic components: (A) Membrane staining, (B) SPB, (C) microtubules, (D) cell pole and septum, (E) cell periphery, and (F) cytoplasmic dots. Asterisks indicate conjugated zygotes. Inset of TA69 in (B) shows two time-lapse images of the same cell; note that the GFP dot indicated by the arrow changes its position as the horse-tail nucleus moves. Left two panels of B507 in (E) shows two focal planes at the middle and apical sides of the same cell. Scale bar represents 10 μm.

were found in many of the gene products in this category (Table 3). A few instances were found in which normally non-nuclear proteins localized to the nucleus in this study, presumably due to C-terminal truncation of the protein and loss of the proper localization signal: D371, containing the N-terminal 142 residues of pap1 protein, exhibited bright nuclear staining, although pap1 protein is a transcription factor localized to the cytoplasm and imported into the nucleus in response to oxidative stress (Toda *et al.* 1991; Kudo *et al.* 1999). A putative bipartite NLS was found in D371 (Table 3) but the identified nuclear export signal located near the C-terminus (Kudo *et al.* 1999) was missing. Another example, TA76, contains the N-terminal 393 residues of ssp1 protein, and a putative bipartite NLS was found in this portion (Table 3). Since ssp1 protein is a protein kinase localized to the cytoplasm and required for actin localization and stress response (Matsusaka *et al.* 1995; Rupes *et al.* 1999), its cytoplasmic localization is likely to be regulated by unidentified sequences at the C-terminal truncated region.

Examples of nuclear dot staining are shown in Fig. 2B for H272, SB44 and TA73: in this category various numbers of distinct dots are observed inside the nucleus. One of them, H272, showed an interesting localization within the cell: in the mitotic interphase, it is typical for 3–4 dots locate to the periphery of nucleus, while in the meiotic prophase, one dot locates to the leading edge of the moving horse-tail nucleus and one or two other dots locate to the rear side of the horse-tail nucleus (Fig. 2B). In addition to the nuclear dots, H272 stains mitotic and meiotic spindle microtubules (indicated by an arrow in Fig. 2B), but not cytoplasmic microtubules. A sequence analysis shows that H272 contains the N-terminal 286 residues of the 601-residue ORF of the uncharacterized gene, SPAC17H9.06c. We also made a chromosomal integration of a GFP-SPAC17H9.06c fusion construct with the GFP gene fused to the 3′-end of the SPAC17H9.06c gene; this full-length fusion construct on the chromosome showed a localization pattern identical to that of H272 (data not shown). Another clone, TB04, exhibited a pattern of nuclear dots similar to that of H272, but TB04 staining was exclusively nuclear and did not stain microtubules (Table 2; image data not shown). TB04 contains the N-terminal 525 residues of the 736 residue gene SPCC830.03. The C-terminal fusion of GFP to the full-length SPCC830.03 on the chromosome reproduced a pattern of staining of TB04 (data not shown).

There are several ribosomal proteins found in nucleolus (Table 2; image data not shown). Examples of nucleolar staining are shown in Fig. 2C for TC37, TA78 and Z031. One of them, TC37, is homologous to FK506- and rapamycin-binding protein (FKBP); two kinds of FKBP have been shown to localize to the nucleolus in *S. cerevisiae* (Benton *et al.* 1994; Shan *et al.* 1994).

Several clones with punctate staining on the nuclear rim staining were obtained in our library; examples are shown in Fig. 2D for SB21, TB70 and TC61. While little is known about the components of the nuclear membrane or the nuclear pore complex (NPC) in *S. pombe*, some punctate nuclear rim staining appears to represent NPC components; SB21 and TB70 for example are homologous to *S. cerevisiae* Nup 133 and Nup2, respectively, which are components of the NPC (Loeb *et al.* 1993; Doye *et al.* 1994). TC61 is similar to importin β; two other proteins, SB02 and TC52, also have importin β N-terminal domain signatures and show punctate staining on the nuclear rim that is very similar to TC61 (Table 2; image data not shown).

(ii) Membrane components

This category of GFP-fusion constructs, containing 57 genes (Table 1), stains all membrane structures including the nuclear membrane, the plasma membrane, the putative ER membrane, and other putative vesicle membranes; gene products which localize specifically to the nuclear membrane are categorized into the nuclear rim staining subgroup of nuclear components described in the previous section (Table 2). Some of the proteins in this category turned out to be membrane transporters of proteins, peptides, amino acids, sugars, and cations (Table 2). Examples of general membrane staining are shown in Fig. 3A for D817, TA22, TA51 and Q769. The D817 construct contains the N-terminal 275 amino acid residues of P450 cytochrome reductase (Miles 1992), within which a single segment of potential transmembrane sequences (Klein *et al.* 1985) is contained. D817 predominantly stains the nuclear membrane and the plasma membrane (Fig. 3A), and is feasible for a marker of the nuclear membrane (Tange *et al.* 1998; also see Fig. 4). TA22 and TA51 also contains five and one potential transmembrane segments, respectively. Q769 contains no obvious integral transmembrane sequences but has 'peripheral' transmembrane sequences defined in PSORT II, and stains general membranes including putative cytoplasmic vesicular membranes.

One of the gene products in this category, TA61, has high similarity to *S. cerevisiae* POM152, an integral protein of the membrane domain of the NPC (Wozniak *et al.* 1994). The full-length POM152 contains a potential transmembrane segment at its N-terminus

Table 2 Intracellular localization categories of 250 GFP-fusion constructs

Clone*	Accession no.	Gene/ORF	GFP fusion position†	Note	References‡
Nucleus					
A213	AB027768	SPAC18B11.10	101/614	hypothetical Trp-Asp repeats containing protein related to *S.pombe* tup1+	
A691 (Fig. 2A)	AB027769	SPBC1685.08	179/442		
B482	AB027773				
B934	AB027776	ste11+	101/468	transcription factor	Sugimoto 1991; Leupold 1991
D371	AB027778	pap1+	142/544	AP-1-like transcription factor	Toda 1991
E183	AB027781	SPBC16A3.19	227/272		
E775	AB027783	SPBC215.03c	133/422	putative signal transduction pathway component	
E961	AB027785				
F288	AB027786		405/479		
F293	AB027787	SPAC24C9.05c	535/730		
F964	AB027789	pyp2+	436/711	protein-tyrosine phosphatase	Ottilie 1992; Millar 1992
F969	AB027790	SPAC2E11.05c	106/727		
G703	AB027792	SPBC19C7.10	381/445		
G737	AB027793	SPBC947.12	153/457	similar to S. pombe kms1+	
H644	AB027796	pol3+	454/1084	DNA polymerase delta large chain	Pignede 1991
H782	AB027798	SPBP8B7.23	158/673	zinc finger protein	
I633	AB027804				
J972	AB027807	SPBC428.17c	111/602	hypothetical serine rich protein	
K026	AB027808	SPBC530.05	70/743	putative transcriptional activator	
L197	AB027814	SPBC29A3.13c	188/359		
L391	AB027815	SPAC27F1.09c	252/1205	putative nuclear protein	
M171	AB027818	SPAC16A10.01	110/830		
M730 (Fig. 2A)	AB027819	SPBC16H5.10c	728/735	putative pre-mRNA splicing factor RNA helicase *S. cerevisiae* PRP43	
SA03	AB027839				
SA04	AB027840				
SA08	AB027842				
SA21	AB027844				
SA29	AB027847	SPAC6F12.16	966/1117	putative helicase	
SA32	AB027848	SPAC630.09c	225/277		
SA50	AB027850	hba1+	359/399	brefeldin A resistance protein	Turi 1996
SA54	AB027851	SPAC6F12.02	364/567	zinc finger protein	
SA68	AB027852	hrp1+	1015/1367	SNF2/SWI2 helicase-related protein	Jin 1998
SB08	AB027858	SPAC637.12c	441/463	putative histone acetyl transferase	
SB29	AB027862	rnp24+	196/347	RNA-binding protein	VanHoy 1996
TA02	AB027866				
TA13 (Fig. 6)	AB027869	cdc13+	226/482	G2/mitotic-specific cyclin	Hagan 1988; Booher 1988
TA14	AB027870				
TA24	AB027876	rhp16+	716/854	nucleotide excision repair protein	Bang 1996
TA27	AB027879	SPAC6B12.16	343/344		
TA29	AB027880	SPCC550.02c	328/354	RNA binding protein	
TA35	AB027882	SPAC31A2.07c	168/848	putative ATP-dependent RNA helicase	

Table 2 (*Continued*)

Clone*	Accession no.	Gene/ORF	GFP fusion position†	Note	References‡
TA36	AB027883	pla1+	436/566	polyA polymerase	Ohnacker 1996
TA40	AB027885	SPCC645.13	199/721		
TA45	AB027889	cdc10+	309/767	'start' control protein	Aves 1985
TA47	AB027891				
TA49	AB027893	SPAC3A11.08	412/734	cullin homolog	
TA54	AB027897	SPAC17G6.12	359/767	putative cell division control protein	
TA66	AB027904	SPAC57A7.06	333/929		
TA70	AB027908	SPAC2E12.01	398/447		
TA75	AB027912	SPAC26A3.09c	301/1275		
TA76	AB027913	ssp1+	393/652	Ser/Thr protein kinase	Matsusaka 1995
TA86	AB027919	SPAC2C6.16	810/997	contains ATP/GTP-binding site motif A (P-loop)	
TA87	AB027920	SPAC23E2.02	404/1273		
TA88	AB027921	SPAC5D6.02c	161/300		
TA90	AB027923	SPBC15D4.09c	213/610	putative cystathionine gamma-synthase	
TA96	AB027926				
TA98	AB027927				
TA99	AB027928	SPBC14F5.08	374/376	possible component of RNA PolymeraseII transcriptional regulation mediator complex	
TB14	AB027934				
TB19 (Fig. 5)	AB027936	pol1+	190/1405	DNA polymerase alpha	Damagnez 1991
TB20	AB027937				
TB23	AB027939	SPCC126.14	236/343		
TB26	AB027940	SPBC24E9.10	620/695		
TB30	AB027942	SPBC31F10.16	415/679		
TB32	AB027944	SPBC3B9.02c	321/381		
TB40	AB027947	SPAC25A8.01c	215/922	putative helicase	
TB46	AB027948				
TB57	AB027952	SPCC70.01	596/964		
TB58	AB027953				
TB68	AB027956				
TB72	AB027959	cut3 +	486/1324	chromosome segregation protein	Saka 1994
TB73	AB027960	pi048	95/351	similar to *S. pombe* alpha-1.2-galactosyltransferase	
TB76	AB027961	SPAC19G12.14	446/742	probable phosphatidylinositol-4-phosphate5-kinase	
TB85	AB027964				
TB93	AB027969	SPAC1D4.01	115/285		
TC02	AB027972	cut15+	199/542	putative importin alpha	Matsusaka 1998
TC10	AB027975				
TC13	AB027977				
TC18	AB027980				
TC19	AB027981				
TC22	AB027983	SPBC17D11.06	248/459	similar to *S. cerevisiae* DNA primase large subunit	
TC23	AB027985	prp2+	168/517	Splicing factor U2AF 59 KD subunit	Potashkin 1993
TC26	AB027987				

Table 2 (*Continued*)

Clone*	Accession no.	Gene/ORF	GFP fusion position†	Note	References‡
TC31	AB027988	SPAC17A5.06	313/804	putative DNA repair helicase	
TC35 (Fig. 2A)	AB027990	SPBC19G7.13	384/458	putative DNA binding factor	
TC40	AB027992	SPBC31F10.13c	920/932	putative histone transcription regulator	
TC41	AB027993	SPBC2F12.03c	288/891	serine-rich protein	
TC48	AB027997	SPBC14C8.14c	795/959	DNA polymerase V	
TC49	AB027998				
TC53	AB028000	SPAC1327.01c		putative transcriptional regulator	
TC62	AB028003	pi029	463/833		
TC71	AB028008	SPAC17G8.03c	172/199		
TC82	AB028014				
TC92	AB028016	SPBC3D6.11c	165/269	transcription factor, putative zinc finger protein	
TC98	AB028017	SPAC23C4.19	351/990	probable involvement in transcription initiation	
U648	AB027829	sad1+	156/514	spindle pole body associated protein	Hagan 1995
U706	AB027830	SPAC22E12.11c	151/859		
V125	AB027831				
Nuclear dots					
G140	AB027791	ufd2+	571/930	putative ubiquitin fusion degradation protein	
H272 (Fig. 2B)	AB027794	SPAC17H9.06c	286/601		
SB44 (Fig. 2B)	AB027864				
TA41	AB027886				
TA60	AB027900	wee1+	625/877	mitosis inhibitor protein kinase	Russell 1987
TA71	AB027909	mcs4+	217/522	mitotic catastrophe suppressor	Cottarel 1997
TA73 (Fig. 2B)	AB027911	SPBC23E6.09	836/1102	probable involvement in transcription	
TB04	AB027930	SPCC830.03	525/736		
TB36	AB027946				
TB67	AB027955	SPCC330.04c	258/357		
Nucleolus					
L452	AB027816				
SA23	AB027845		47/253	60s Ribosomal protein k5	Gatermann 1989
SA89	AB027855	SPBC3D6.15	52/92	40s ribosomal protein s25	
SB46	AB027865	SPAC2E12.02	222/609	heat shock factor protein (HSF)	Gallo 1993
TA09	AB027868	SPBC3B8.09	461/597		
TA100	AB027929		66/191	probable 40s ribosomal protein s9	
TA38	AB027884	SPAC926.08c	278/317		
TA57	AB027899	SPAC29A4.10	537/556		
TA78 (Fig. 2C)	AB027915				
TA82	AB027916	SPAC4F10.07c	302/758		
TA93	AB027925	SPAC13G7.03	224/278		
TB05	AB027931	SPBC23E6.07c	645/934	replication factor 3 like protein	
TB27	AB027941	SPBC2D10.10c	293/305	fibrillarin	Girard 1993
TB31	AB027943	SPAC8C9.14	413/539	putative transcription factor	
TB88	AB027965	SPAC19G10.02	443/830	putative helicase	
TB95	AB027970	SPAC8A4.13c	339/594		
TC16	AB027979	SPBC19F5.05c	116/607		

Table 2 (*Continued*)

Clone*	Accession no.	Gene/ORF	GFP fusion position†	Note	References‡
TC37 (Fig. 2C)	AB027991	SPBC1347.02	145/361	FKBP-type peptidyl prolyl cis-trans Isomerase	
TC42	AB027994	SPAC13G6.02c	72/252	40s ribosomal protein RP10	
TC65	AB028005	SPAC6F6.03c	104/537	hypothetical GTP-binding protein associated	
TC74	AB028010	SPBC887.03c	104/747		
Z031 (Fig. 2C)	AB027836	SPAC1B3.09c	372/528		
Nuclear rim					
I226	AB027801	phz1+	26/515	PPZ protein phosphatase	Balcells 1997
I596	AB027803				
J440	AB027826		53/257	ATP synthase A chain	Massardo 1990
K028	AB027809	SPAC19D5.02c	153/223		
SA02	AB027838	SPBC30B4.05	835/844		
SB02	AB027856	SPBC30B4.05	166/967	putative chromosomal segregation protein, has an importin beta N-terminal domain motif, similar to *H. sapiens* CAS	
SB21 (Fig. 2D)	AB027860	SPAC1805.04	1055/1162	putative nuclear envelope pore protein; similar to *S. cerevisiae* NUP133	
TA26	AB027878	SPBC3H7.02	646/877	sulfate permease	
TA43	AB027888				
TB11	AB027933	SPBC1685.14c	609/803		
TB70 (Fig. 2D)	AB027958	SPCC18B5.07c	485/565	putative nucleoporin; similar to *S. cerevisiae* NUP2	
TC14	AB027978	SPAC22G7.02	727/990		
TC52	AB027999	SPCC1840.03	307/1095	putative importin beta	
TC61 (Fig. 2D)	AB028002	SPBC14F5.03c	605/1067	putative importin beta-4	
TC88	AB028015	SPAC23H4.01c	608/749	probable involvement in sterol metabolism	
Membrane					
A762	AB027770	itr1+	280/575	myo-inositol transporter	
B759	AB027775		44/48	ATP synthase protein	
C378	AB027777	pho4+	71/463	thiamine-repressible acid phosphatase	Yang 1990
D817 (Figs 3A,4)	AB027780	SPBC365.17	275/678	NADPH-cytochrome P450 reductase	Miles 1992
E626	AB027782	sec61+	178/479	protein transport protein Sec61 alpha subunit	Broughton 1997
F961	AB027788				
H717	AB027797	SPAC2F3.08	165/553	putative sucrose carrier	
J759	AB027805				
J794	AB027806	sxa1+	480/533	aspartic proteinase	Imai 1992
L505	AB027817				
P592	AB027820	SPAC9E9.04	109/188		
Q009	AB027821				
Q769 (Fig. 3A)	AB027822	SPAC25H1.07	445/885		
R934	AB027823	SPCC1919.03c	116/298		
SA05	AB027841	SPAC2C4.05	70/134	cornichon homolog	
SA18	AB027843	SPAC30D11.01c	286/993	putative family 31 glucosidase	

Table 2 (*Continued*)

Clone*	Accession no.	Gene/ORF	GFP fusion position†	Note	References‡
SA28	AB027846	SPBC1734.12c	311/547	putative involvement in cell wall structure or biosynthesis	
SA33	AB027849	SPAC24H6.13	782/871		
SA78	AB027853	SPAC2E11.07c	809/1211	putative cation transporting ATPase	
SA82	AB027854				
SB04	AB027857				
SB16	AB027859				
SB25	AB027861	SPAC23D3.12	411/559	putative inorganic phosphate transporter	
SB40	AB027863				
TA20	AB027874				
TA22 (Fig. 3A)	AB027875	SPAC24B11.12c	855/1402	Ca transporting ATPase	
TA31	AB027881				
TA51 (Fig. 3A)	AB027895	isp4+	143/767	sexual differentiation process protein	Sato 1994
TA52	AB027896	SPAC23H4.07c	195/227	probable involvement in protein targeting	
TA61	AB027901	SPBC29A10.07	547/1250	putative nuclear envelope pore membrane protein; similar to *S. cerevisiae* POM152	
TA67	AB027905				
TA77	AB027914	SPBC20F10.07	729/764		
TA83	AB027917				
TA84	AB027918				
TA89	AB027922				
TA92	AB027924				
TB07	AB027932	SPAC17G8.11c	84/356		
TB18	AB027935	fnx1+	462/531	multidrug resistance protein	Dimitrov 1998
TB35	AB027945				
TB53	AB027949	pho1+	285/453	acid phosphatase	Elliott 1986
TB54	AB027905				
TB56	AB027951	SPBC3E7.06c	238/577	major facilitator family transporter	
TB60	AB027954				
TB69	AB027957	SPBC24E9.08c	384/935		
TB80	AB027962				
TB89	AB027966				
TC07	AB027973				
TC08	AB027974	SPAC26H5.07c	266/424	putative mannosyl transferase	
TC12	AB027974				
TC25	AB027986	SPBC16C6.09	105/778	dolichyl-phosphate-mannose–protein o-mannosyl transferase	
TC46	AB027996	SPAC23C11.06c	159/535		
TC64	AB028004	SPAC6G10.09	531/808	probable mannosyl-oligosaccharide glucosidase	
TC73	AB028009	SPBC1773.11c	127/396	putative *S. cerevisiae* cell division cycle CDC50 homolog	
TC78	AB028011	SPBC1734.04	414/430	putative involvement in protein glycosylation in the golgi	
U120	AB027828	SPAC14C4.09	39/402		
X496	AB027834	SPBC30B4.01c	35/344		
X571	AB027835	SPBC36.01c	343/580	putative membrane transporter	

Table 2 (*Continued*)

Clone*	Accession no.	Gene/ORF	GFP fusion position†	Note	References‡
SPB					
TA69 (Fig. 3B)	AB027907	SPAC19A8.02	726/1213		
TB98 (Fig. 3B)	AB027971				
TC80 (Fig. 3B)	AB028012		281/1173	coiled-coil protein, myosin-like	
Microtubule					
A937 (Fig. 3C)	AB027772	SPBC25B2.07c	293/501	serine proline rich protein	
I450 (Fig. 3C)	AB027802				
K360	AB027811	SPAC1F3.06c	870/1957		
TA65 (Fig. 3C)	AB027903	SPBC1604.20c	545/628	putative kinesin motor	
TA68 (Fig. 3C)	AB027906	pkl1+	464/784	kinesin-like protein	Pidoux 1996
Cell pole/Septum					
D510	AB027779	SPAC4F8.13c	531/1489	ras GTPase-activating-like protein	
TA42	AB027887	SPAC29A4.11	969/969		
TB90 (Fig. 3D)	AB027967	SPBC1734.17	925/926	putative chitin synthase	
TC20 (Fig. 3D)	AB027982	pck1+	535/988	protein kinase C-like protein	Toda 1993
TC22N	AB027984				
TC58	AB028001	SPCC1919.10c	1382/1516	putative myosin heavy chain	
TC67	AB028006	cdc12+	689/1841	cell division control protein	Chang 1997
W209	AB027832	SPAC31A2.14	948/962	hypothetical Trp-Asp repeats containing protein	
Cell periphery					
B507 (Fig. 3E)	AB027774	SPAC1A6.07	297/636		
S644	AB027824	pck2+	927/1016	protein kinase C-like protein	Toda 1993
TA16	AB027872	SPAC27F1.01c	281/1794	similar to *S. cereviciae* PAN1	
TA17	AB027873	SPAC10D6.07c	414/421		
TA46	AB027890	SPBC3D5.14c	280/309		
TA50	AB027894	SPAC13G7.04c	442/756		
TA56	AB027898	SPAC17G6.02c	314/324	similar to *S. cerevisiae* RTM1 protein; resistance to molasses	
TA63	AB027902	SPCC70.05c	545/781	Ser/Thr protein kinase	
TC45 (Fig. 3E)	AB027995				
TC69	AB028007		295/415		
TC81	AB028013	aap1+	557/594	amino acid permease	
Cytoplasmic structures					
E906	AB027784	SPAC12G12.03	569/576		
H461	AB027795	SPBC1709.17	125/505	folypolyglutamate synthase	
I040	AB027799	SPAC6F12.10c	1121/1323	hypothetical phosphoribosyl-formylglycinamidine synthase	
I096	AB027800	pss1+	305/720	heat shock protein 70-like protein	Chung 1998
K294	AB027810	cdc15+	334/907	cell division control protein	Fankhauser 1995
K705	AB027812	myp2+/myo3+	2077/2104	the second myosin II/type II myosin	Bezanilla 1997; Motegi 1997
K755	AB027813	dak1+	167/591	dihydroxyacetone kinase	Kimura 1998
S866	AB027825	SPAC2C4.07c	659/927	ribonuclease II RNB family protein, similar to *S. pombe* Dis3	
TA08	AB027867				
TA15	AB027871	sum3+	339/636	suppressor of uncontrolled mitosis	Forbes 1998
TA25 (Fig. 3F)	AB027877	SPBC1734.13	125/301	ATP synthase gamma chain, mitochondrial precursor	

Table 2 (*Continued*)

Clone*	Accession no.	Gene/ORF	GFP fusion position†	Note	References‡
TA48	AB027892	SPAC5H10.14	492/647		
TA72	AB027910	SPCC794.10	253/499	putative UTP–glucose-1-phosphate uridylyltransferase	
TB22	AB027938				
TC32	AB027989	SPCC14G10.01	182/236		
TC99	AB028018	SPAC13G7.13c	385/533		
U017 (Fig. 3F)	AB027827	SPCC1795.10c	189/227		
X267	AB027833	SPAC23C11.18c	630/780	DNA mismatch repair muts family	
Z651	AB027837	ntp1+	358/735	neutral trehalase	Soto 1998
Spore rim					
A799	AB027771	SPAC17A5.04c	49/512	probable metallopeptidase, has TR-Box in promoter region	
TB91	AB027968				

*Listed by clone names alphabetically and numerically in each localization category.
†Number of amino acid residues contained in the GFP-fusion construct/total number of amino acid residues in the full-length ORF.
‡References for *S. pombe* genes. Cited by the first author and publication year.

(residues 88–104) and a bipartite NLS at its C-terminus (residues 725–741; Table 3), and is expected to show a punctate nuclear membrane localization. On the other hand, TA61 comprises the N-terminal 547 residues, containing the transmembrane segment but lacking the NLS, appears to lose the specific localization to the NPC and localizes to general membranes.

(iii) Cytoskeletal and cytoplasmic components

The remaining gene products were localized to the SPB, microtubules, cell poles, septum and cell periphery (Tables 1 and 2). Three gene products, TA69, TB98 and TC80, none of which have been previously documented, were localized primarily to the SPB (Fig. 3B); note that the SPB is located at the leading edge of the moving, elongated nucleus in meiotic prophase. In addition to the SPB staining, TA69 stained the cell poles, septum and mitotic spindle, and the meiotic conjugation site. TB98 and TC80 also stained microtubule organizing centres other than the SPB, and additionally, they both stained the nuclear envelope. This may be a similar situation in which a well-characterized SPB component, sad1 protein, also localizes in the nuclear membrane when expressed on a multicopy plasmid (Hagan & Yanagida 1995). Five gene products were localized to microtubules (Table 2; Fig. 3C), including two kinesin-related proteins (TA65 and TA68). It should be noted that one of them, I450, also stained the nucleus; in fact, a putative NLS was found in I450 (Table 3). Eight gene products localized to the cell pole and septum (Table 2; Fig. 3D), 11 gene products localized to the cell periphery (Table 2; Fig. 3E), 19 gene products localized to cytoplasmic structures (Table 2; Fig. 3F), and 2 gene products localized to the spore rim (Table 2; image data not shown).

Live observation of GFP-fusion constructs

Several GFP-fusion constructs can be used as fiduciary fluorescent markers for intracellular structures, to follow their behaviour in living cells. An example using D817 as a marker of the nuclear membrane is shown in Fig. 4. The images in Fig. 4 illustrate how D817 manifests the changes which occur in the nuclear membrane during mitosis (Fig. 4A) and meiosis (Fig. 4B).

Certain constructs can also be used to monitor cell cycle stages. A straightforward example is shown in Fig. 5 using TA13. This construct contains the N-terminal 226 amino acid residues of cyclin B (cdc13 gene product), including the destruction box (Booher & Beach 1988). Figure 5 shows the intensity of nuclear fluorescence in TA13, mirroring the characteristic changes associated with cyclin B expression observed during the mitotic and meiotic cell cycles. In the mitotic cell, nuclear fluorescence reached a maximum at metaphase and decreased at the onset of anaphase (Fig. 5A; note the rounded nucleus at hour 3.4 and the dividing nucleus in the next frame). In the meiotic cell, nuclear fluorescence reached a maximum

Table 3 Putative NLS sequences

NLS type	Clone	Putative NLS sequence	Note	Localization††
Bipartite type *	D371	**KK**IGRKNSDQWPSS**KRK**	pap1+	A
	E961	**KK**KSFSSMLAQV**KK**E**K**A		A
	F288	**KR**RALARRNSLA**RRR**SN		A
	G737	**RK**SVAKPQKISEN**R**I**KR**K	SPBC947.12	A
	H644	**RR**NGSIHGEITDV**KRRR**	pol3+	A
	H782	**RK**NQQLASSERKT**K**N**KK**R	SPBP8B7.23	A
	J972	**KR**SPGQTVSKRLH**KK**Q**R**	SPBC428.17c	A
	K026	**KR**IPRACDMCRK**RK**I**R**C	SPBC530.05	A
	L391	K**KR**ELELNNTEISQ**K**P**KR**	SPAC27F1.09c	A
	L452	**RR**VDGASNVTQDY**KR**A**K**		C
	SA02	**KK**AQLITLFSKL**RR**A**K**N	SPAC14C4.05c	D
	SA04	**KR**RTKTGCLTCR**RRR**I		A
	SA29	**RK**LALLEEVKDL**KKK**LS	SPAC6F12.16	A
	SA68	**KK**SVASDDEDAYD**KR**H**R**	hrp1+	A
	TA24	**KK**VDSLSMVRRT**K**LE**RR**	rhp1+	A
	TA29	**KK**VEKRELHSRPP**KRK**L	SPCC550.02C	A
	TA40	K**RR**GSVGTTATHT**KR**S**K**N	SPCC645.13	A
	TA76	**KR**LQEWGEVKET**KK**I**RK**R	ssp1+	A
	TB05	**KK**PKVSPTPTSP**K**P**KR**S	SPBC23E6.07c	C
	TB14	**RK**GGPRSGVGSR**KRKR**A		A
	TB20	**RK**ANALAKKNDKI**KKK**D		A
	TB23	K**RR**QLEGTSELPV**KK**AF**R**R	SPCC126.14	A
		KKAFRRGDWEKE**R**E**KK**Y		
	TB26	**RK**DQLEAEEYYA**R**E**RR**	SPBC24E9.10	A
	TB46	**KR**GKDFAMRRAF**R**F**R**G**R**		A
	TB58	**RKRR**GSVGTTATHT**KR**S**K**N		A
	TC02**	**RR**RRETQQIEIR**K**Q**KR**E	cut15+	A
	TC18	**KR**ISYHTEYLRL**R**Q**KR**L		A
	TC41	**KKKR**NKEALWLRAR**Q**K**K**W**K**	SPBC2F12.03c	A
	TC48	**RR**QWATDTMLSIL**R**S**KR**	pol5+	A
	TC49	**RR**TYSSISSSSSPF**KKK**		A
	TC53	**RKR**LPLACQSCR**KKK**V**K**	SPAC1327.01c	A
	Z031	**KK**FTKNHLKNTI**E**R**RK**Q	SPAC1B3.09c	C
Bipartite type				
SV40 T (5R/Ks) †	SB29	K**KK**EEKRLKRLDA**K**Y**GRK**	rnp24+	A
		KKRKK		
	TA70	R**RR**KKLLPSQRGG**KKKSK**	SPAC2E12.01	A
		RRRKK		
	TA88	**RK**PEIVKPTLRK**R**G**RK**P	SPAC5D6.02c	A
		RRKRRK		
	TB19	**KK**GNRSHTTSNA**KRR**SQ	pol1+	A
		RKRKKSK		
SV40 T (5R/Ks) ‡	H272	**RKKRK, KRKRK**	SPAC17H9.06c	B
	SA54	**RRRRR**	SPAC6F12.02	A
	SB08	**KKRRR**	SPAC637.12c	A
	TC23	**RRRRR**	prp2+	A
SV40 T (4R/Ks) §	SB44	**RRKK**		B
	TA13	**PKKR, P**AS**KKRR, PKK**L**KK**D	cdc13+	A
	TA66	**KRKK**	SPAC57A7.06	A
	TA86	**KRKR**	SPAC2C6.16	A

Table 3 *(Continued)*

NLS type	Clone	Putative NLS sequence	Note	Localization††
	TA96	**RKRK**		A
	TB76	**RRRR**	SPAC19G12.15	A
	TB93	**RRRK**, **KRRK**	SPAC1D4.01	A
	TC26	**RKRK**		A
	TC62	**KKKK**	pi029	A
	U706	**RRRR**	SPAC22E12.11c	A
SV40 T (others)¶	B482	**PKR**W**R**SS, **PPKK**S**RP**		A
	B934	**PKKR, RRRHKK**	ste11+	A
	F293	**PVRKIRR**	SPAC24C9.05c	A
	I450	**HRKR**, **P**EF**KHRK**		E
	M171	**P**EES**RKR**	SPAC16A10.01	A
	SA89	**PKKK**WS**K**	SPBC3D6.15	C
	TA09	**PYKERR**F	SPBC3B8.09	C
	TA45	**KRHR**	cdc10+	A
	TA49	**PRK**S**R**Q**R**	SPAC3A11.08	A
	TA38	**KKPK, PKPKK**NV	SPAC926.08c	C
	TB88	**P**L**PKKKH**SI**K, KKKP**	SPAC19G10.02	C
	TB72	**RPRK**	cut3+	A
	TC16	**PK**N**KKK**A	SPBC19F5.05c	C
	TC71	**KRPR, P**VA**RIKK, PR**A**RR**AA, **PAKK**V**KK**	SPAC17G8.03c	A

Proteins are targeted to the nucleus by specific nuclear localization signals (NLSs) within the proteins' primary sequence. Among the known nuclear targeting sequences there are no strict consensus NLSs, but some general features have been found to be conserved (Dingwall & Laskey 1991; Garcia-Bustos *et al*. 1991; Hicks & Raikhel 1995). One type of NLS is a single cluster of basic amino acids such as that found in the large T antigen of SV40. Another type is the 'bipartite' NLS found in nucleoplasmin, two clusters of basic amino acid separated by a spacer region of any ten amino acids (Robbins *et al*. 1991). Here, we listed the putative NLS sequences (Nakai & Kanehisa 1992) found in the GFP fusion proteins which localized to the nucleus.

*This group contains a bipartite type NLS: comprised of two basic amino acids followed by a spacer region of any ten amino acids and then a group of five amino acids of which at least three must be basic.

†This group contains both the bipartite type NLS seen in the first group and, additionally, a single cluster of five basic amino acids (R or K).

‡This group contains at least one cluster of five basic amino acids (R or K).

§This group contains at least one cluster of four basic amino acids (R or K).

¶This group contains at least one cluster of the 'pat4' or 'pat7' sequences defined in the PSORT II program <http://psort.ims.u-tokyo.ac.jp>.

Cut15+ is the *S. pombe* homolog of *Xenopus* importin alpha. The amino acid sequence of cut15+ corresponding to the reported NLS sequence in importin alpha is RRQNYKGKGTFQADELRR**RRETQQIEIR**KQKRE**ENLNKRRNL and contains a typical bipartite NLS.

††Intracellular localization categorized in Table 2: A, nucleus; B, nuclear dots; C, nucleolus; D, nuclear rim; E, others.

at the first meiotic metaphase when the nucleus stops the horse-tail nuclear movements (Fig. 5B, frames 1 h 40min–2 h 20min) and disappeared at the onset of the first meiotic anaphase (Fig. 5B, 2h 30min); during the second meiotic division, its intensity increased toward the second meiotic metaphase (Fig. 5B, 2h 50min–3 h 20min) and disappeared at the onset of the second meiotic anaphase (Fig. 5B, 3h 30min).

A second example of observing a cell cycle-specific nuclear marker is shown in Fig. 6 using TB19. This construct contains the N-terminal 190 amino acid residues of DNA polymerase α; TB19 contains putative NLSs (Table 3), but lacks the catalytic domain of the enzyme (Damagnez *et al*. 1991). The intensity of nuclear fluorescence in TB19 also showed characteristic changes during the mitotic and meiotic cell cycles. In the mitotic cell, nuclear fluorescence reached a maximum just prior to cell separation, which corresponds to S phase, and declined thereafter (Fig. 6A). Thus, these two constructs TA13 and TB19 act as a cell cycle-specific nuclear marker for G2/M phase and S phase, respectively.

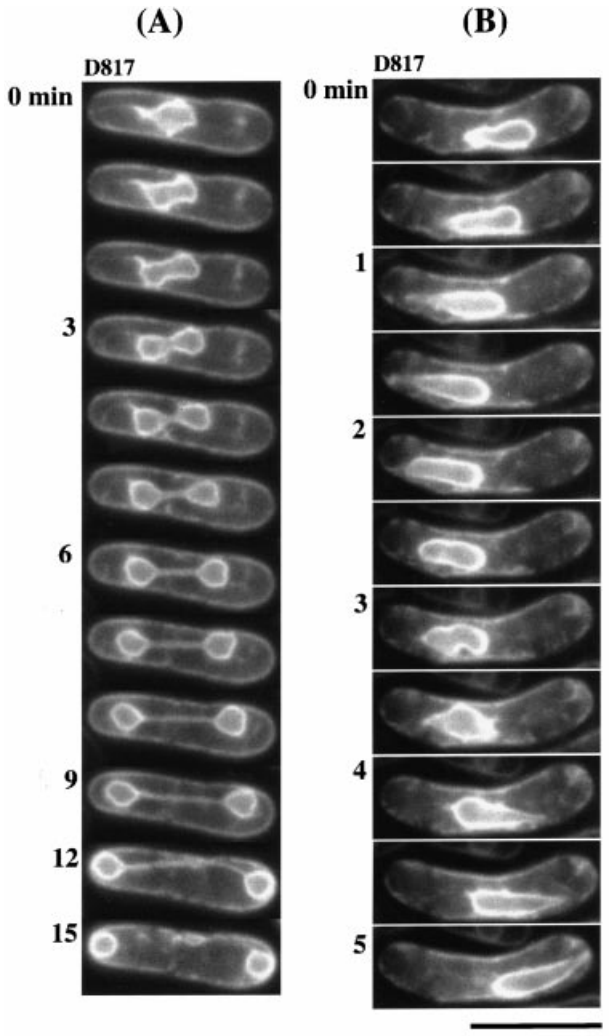

Figure 4 Live observation of the nuclear membrane. (A) Mitotic nucleus, and (B) horse-tail nucleus observed in living cells of the D817 transformant. The horse-tail nucleus in (B) shows oscillatory movement between the cell poles. Numbers on the left represent time in minutes. Scale bar represents 10 μm.

In the meiotic cell, TB19 nuclear fluorescence can be detected during karyogamy (Fig. 6B, 0 min–20 min), but is especially bright during the first half of the nuclear movements (Fig. 6B, 1 h 01 min–2 h 01 min) and gradually becomes dim toward the first meiotic division (Fig. 6B, 3 h 11 min–3 h 32 min); this behaviour reflects the fact that premeiotic DNA replication takes place at the beginning of the horse-tail period in *S. pombe* (Bähler *et al.* 1993). The nuclear fluorescence of TB19 exhibited an interesting characteristic during the second meiotic division, with fluorescence increasing again after the first meiotic nuclear division (Fig. 6B, 3h 37 min–3 h 52 min) and disappearing toward the second meiotic metaphase (Fig. 6B, 4 h 02 min–4 h 37 min). The increase of the TB19 nuclear fluorescence during the second meiotic interphase implicates a pseudo S phase in meiotic nuclear cycles, in which DNA polymerase accumulates in the nucleus but no DNA replication is taking place.

Discussion

We constructed a GFP-fusion genomic DNA library of the fission yeast *S. pombe*. The haploid genome of *S. pombe* consists of 13.8 Mbp of DNA (Fan *et al.* 1989), similar to that of the budding yeast *S. cerevisiae* (Goffeau *et al.* 1997). Efficient screening of the genomic DNA was possible in *S. pombe* because of its relatively small genome and the high density of coding regions. In *S. cerevisiae*, the total number of genes is about 6000 and the coding sequences of genes occupy 72% of the genome (Goffeau *et al.* 1997; Sherman 1997). Assuming a similar genomic organization in *S. pombe*, we estimate that our library comprises 50% of the ORFs in the genome if the DNA fragments were cloned randomly (see the Statistics section in Experimental procedures). However, the frequency of genes being cloned in replicate was higher than that expected by random cloning, DNA sequencing of 512 clones identified only 250 genes, indicating that the library is likely biased. It is likely that portion of the uncloned ORFs were excluded from the library for one of the following reasons: the introduced fusion construct was lethal to the transformed cell; a localization signal at the C-terminal region was truncated; the fusion of GFP to the gene product may disturb proper localization; genes with no appropriate cleavage site for the Sau3AI restriction enzyme were not cloned.

In our library, GFP-fusion constructs are expressed on a multicopy plasmid. An obvious limitation of our library is that those gene fragments which abate cell viability can not be obtained. In this context, it should be pointed out that our library is complementary to a GFP-fusion library of *S. pombe* that had been constructed previously (Sawin & Nurse 1996); this library selected for genes which disturbed mitotic growth when expressed, thus complementing our library in which such genes were excluded. Notably, none of the genes reported in Sawin & Nurse (1996) are included in our library.

Another limitation of our library is occasional mislocalization of the fusion gene products. Because we fused GFP to the 3′-end of the gene, in order that the expression of the fusion construct be under the control of the gene's authentic promoter, the C-terminal portion of the gene product was truncated to various extents and replaced by GFP. Gene products that have localization signals at their C-termini could

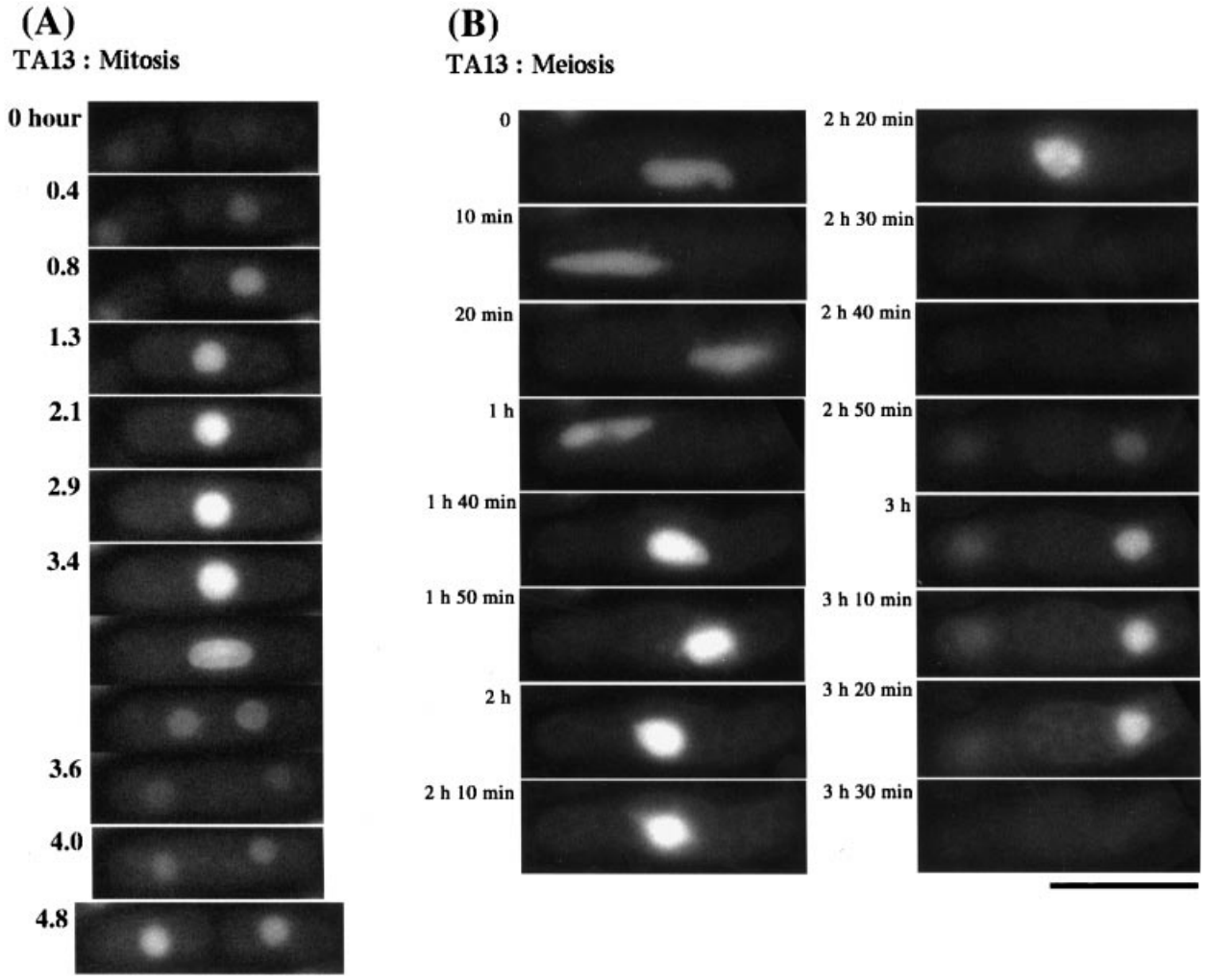

Figure 5 Fluorescent nuclear marker for the G2/M phase. Nuclear fluorescence in mitotic cell cycle (A) and in meiosis (B) observed in living cells of the TA13 transformant. Numbers on the left represent time. Scale bar represents 10 μm.

be mislocalized or excluded during screening. In addition, cryptic localization signals can occasionally be contained within the amino acid sequences fused to GFP.

Finally, it deserves comment that a spacer between GFP and the gene may affect the efficiency of localization of the GFP-fusion protein. We constructed our libraries using three plasmid vectors, each of which fuse the GFP gene in one of three reading frames to the genomic DNA insert (see Experimental procedures). The frame 3 plasmid elicited the best results, in that about 20% of the clones screened had a GFP signal, compared with the frame 1, about 13%, and frame 2, about 11%, plasmids (Table 1). The relative inefficiency of cells transformed with frame 1 and frame 2 plasmids in expressing GFP-fusion proteins may have come from the linker sequence between the GFP gene and the insertion. In frame 3 plasmid three amino acid residues Leu, Gly and Ser were inserted between GFP and the insertion, while in the frame 2 plasmid, three amino acid residues Trp, Gly and Ser were inserted, and in frame 1 plasmids there was no spacer codon between GFP and the insertion. Tethering the GFP directly to the protein backbone or with a spacer made of a heterocyclic aromatic amino acid, tryptophan, may affect the secondary structure of the fusion protein and, consequently, may disturb its intracellular localization.

Conclusions

The GFP-fusion genomic DNA library in which the genes fused to GFP are expressed under their own promoters allowed us to examine the intracellular localization of gene products under physiological conditions both in mitotic

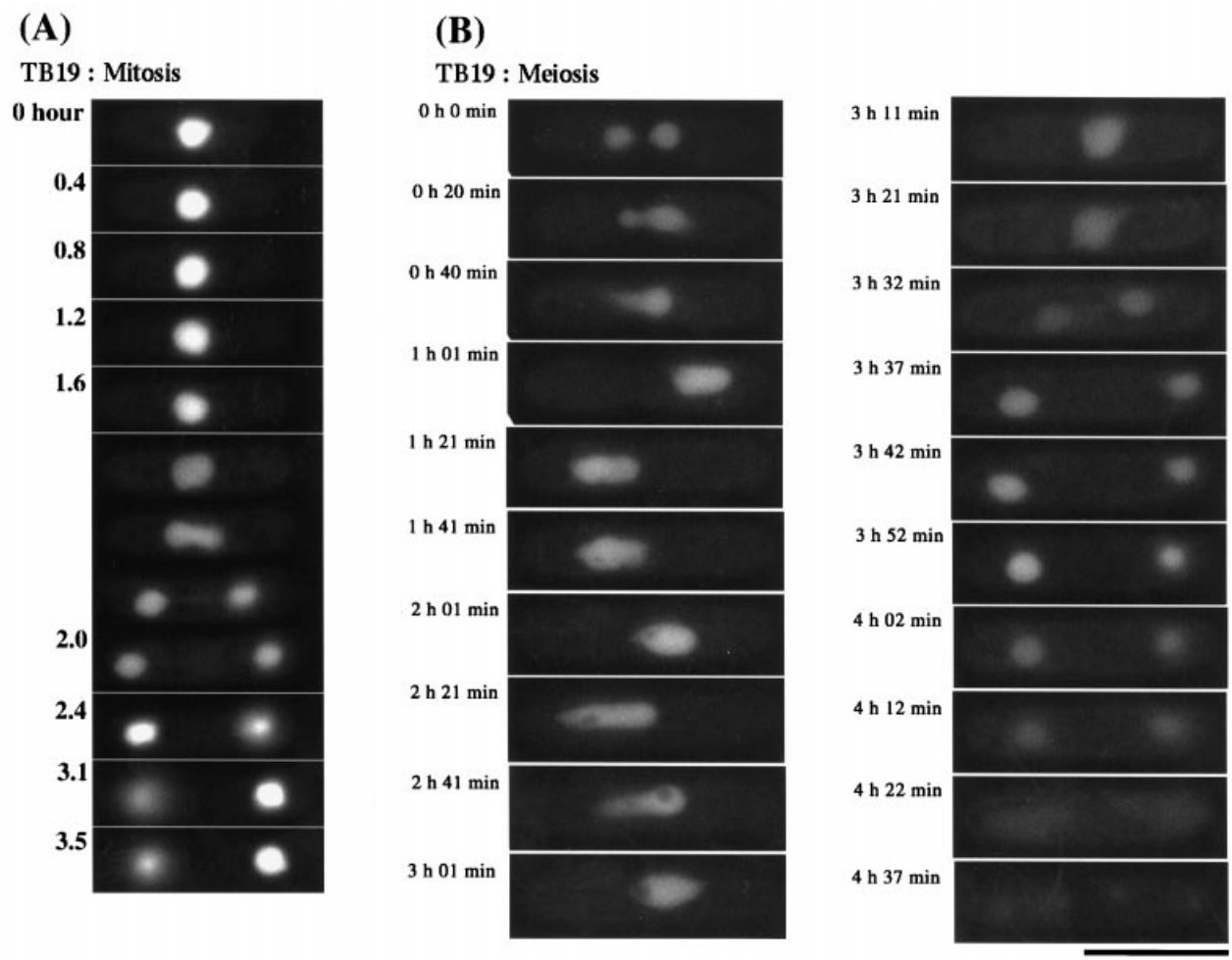

Figure 6 Fluorescent nuclear marker for the S phase. Nuclear fluorescence in mitotic cell cycle (A) and in meiosis (B) observed in living cells of the TB19 transformant. Numbers on the left represent time. Scale bar represents 10 μm.

and meiotic cells. Microscopic screening of this library revealed a number of new components of various intracellular structures. This library provided the foundation for an extensive survey of the intracellular localization of *S. pombe* proteins. Once the *S. pombe* genome project is completed, the intracellular locations of about 50% of the gene products can be searched for using our DNA sequence and image database. The library of GFP-fusion constructs also provides useful fluorescent markers for various intracellular structures and cellular activities, which can be readily used for microscopic observation in living cells in mitosis and meiosis.

Experimental procedures

Microscope system setup

A computer-controlled, fluorescence microscope system employing a cooled, charge-coupled device (CCD) as an image detector was used to obtain fluorescence images. In our microscope system, a Peltier-cooled CCD camera CH250 (Photometrics Ltd, Tucson, Arizona) is attached to an Olympus inverted microscope IMT-2 or IX70; microscope lamp shutter, focus movement, filter combinations, and CCD data collection are controlled by a Silicon Graphics UNIX workstation (Hiraoka *et al*. 1991).

Strains and culture conditions

The *S. pombe* strain 968h^{90} was used to prepare genomic DNA. Homothalic *S. pombe* strains CRL126 (h^{90} leu1–32 ura4) and CRL152 (h^{90} leu1–32 ura4 lys1) were used for transformation with the GFP-fusion genomic DNA library (see below). Complete medium YEade (YE containing 75 μg/mL adenine sulphate) and minimum medium EMM2 were used for routine culture of *S. pombe* strains (Moreno *et al*. 1991). For observation of meiosis, homothalic h^{90} strains cultured in EMM2 supplemented

with appropriate nutrients were washed in EMM2-N (EMM2 deprived of nitrogen sources) and then incubated in EMM2-N at 26 °C.

Construction of the library

Figure 1 summarizes our strategy for the construction and screening of a gene library in which *S. pombe* genomic DNA fragments from the wild-type strain were fused to the 5′-end of the GFP-S65T gene. Parent library plasmids were constructed based on pREP1 (Maundrell 1993). First, the nmt1 promoter region in pREP1 was deleted using the *Pst*I and *Nde*I sites at the ends of the nmt1 promoter, and the resultant nmt1 promoter deleted pREP1 was then digested with *Bam*HI and *Sma*I. The coding sequence of GFP-S65T (Heim & Tsien 1996) was amplified by PCR using GFP-S65T cloned in pRSET-B (a gift of Dr Tsien) as a template, CGCGGATCCCATGAGTAAAGG AGAAGAACTT as the 5′ primer and GAAGGCCTATTTGTA TAGTTCATCCATGCC as the 3′ primer. The PCR products were digested with *Bam*HI and *Stu*I and inserted into the *Bam*HI/*Sma*I digested and nmt1 promoter deleted pREP1 plasmid. The resultant plasmid, pEG3-1, was used as the first frame library plasmid. The second frame plasmid, pEG3–2, was constructed by inserting a 12 base (CCCAGATCTGGG) *Bgl*II linker into pEG3-1 that had been digested with *Bam*HI and blunt ended. The third frame plasmid, pEG3-3, was constructed by inserting a 10 base (CCAGATCTGG) *Bgl*II linker into the blunt ended, *Bam*HI digested pEG3-1. The plasmids pEG3-1, pEG3–2, and pEG3–3 contain the *S. pombe* ars1 and the *S. cerevisiae* LEU2 gene as the selection marker.

Genomic DNA from 968h^{90} was isolated in two ways. For the first frame library, we isolated genomic DNA according to Moreno *et al.* (1991). For the second and third frame libraries, we isolated genomic DNA according to Matsumoto *et al.* (1987), which includes two centrifugation steps during the isolation of nuclei to reduce mitochondrial contamination: the contamination from the mitochondria genome decreased from 20% in the first frame library to 7% in the second and third frame libraries. The isolated genomic DNA was partially digested with Sau3AI, and DNA fragments of 3–6 kbp were concentrated by sucrose gradient centrifugation. The DNA fragments were ligated into *Bam*HI digested pEG3-1 and *Bgl*II digested pEG3-2 and pEG3-3 plasmids and transformed into *E. coli* DH5α. For the frame 1 library, about 22 000 independent DH5α clones were pooled to prepare library DNA. For frame 2 and 3 libraries, about 15 000 and 14 000 independent DH5α clones, respectively, were pooled to prepare library DNA. Examination of randomly selected *E. coli* clones indicated that 84% of frame 1, 78% of frame 2, and 75% of frame 3 clones contained insertions, and that the average length of the insertions was about 4.8 kbp.

Screening of the library

Library DNA was transformed into *S. pombe* cells of a homothalic h^{90} strain, CRL126, using a lithium chloride procedure (Moreno *et al.* 1991). Transformed cells were cultured on plates with minimum medium EMM2 supplemented with 75 μg/mL of adenine, uracil, histidine, and lysine. Single colonies were picked with toothpicks and patched on to new plates. The cells were grown on plates for 24 h at 33 °C and then for 12 h at 26 °C before screening with a fluorescence microscope—the low temperature step appears to enhance both GFP fluorescence and entry to meiosis.

For microscopic screening, cells of independent transformations were suspended in EMM2-N in 10-well immunofluorescence slides (Polysciences), and observed on our CCD microscope system using an Olympus oil immersion objective lens (SPlan Apo 60/NA = 1.4) and high-selectivity excitation and barrier filters for fluorescein (Chroma Technology, Brattleboro, Vermont).

The plasmids in those transformants exhibiting distinct staining patterns of GFP fluorescence were recovered and partial sequences of the DNA inserted at the GFP gene junction point were determined using the DNA sequencer ABI377 (Perkin-Elmer). Transmembrane sequences and NLSs were searched for using the PSORT II program <http://psort.ims.u-tokyo.ac.jp> (Nakai & Horton 1999).

Statistical evaluation of the library

The fraction of the genomic ORFs screened in the library, f, is obtained as $f = 1 - (1 - p/6)^N$, where p is the proportion of the genome occupied by the average ORF, and N is the number of clones screened. Divided by a factor of 3 for the right reading frame and by a factor of 2 for the right direction, $p/6$ gives the probability for a given ORF being fused in-frame to the GFP gene in the right direction.

Although we screened 49 845 *S. pombe* transformants, not all of them contained an insert of genomic DNA. We estimated the number of effective genomic clones as follows: for the frame 1 library, 84% of the *E. coli* clones contained a DNA insert, and 80% of these contained genomic DNA therefore 17 132 of 25 494 *S. pombe* clones ($25\,494 \times 0.84 \times 0.8$) are expected to contain a genomic DNA fragment. Likewise, for the second and third frame libraries, 8766 of 12 084 *S. pombe* clones ($12\,084 \times 0.78 \times 0.93$) and 8556 of 12 267 *S. pombe* clones ($12\,267 \times 0.75 \times 0.93$) are expected to contain a genomic DNA fragment. Summing up the three libraries, we estimate the number of clones containing a genomic DNA insert, N, to be 34 454 out of the 49 845 total clones screened.

In *S. cerevisiae*, the total number of genes is about 6000 and the coding sequences of genes occupy 72% of the genome (Goffeau *et al.* 1997; Sherman 1997). Assuming a similar genomic organization in *S. pombe*, the proportion of the average ORF in the genome $p = 0.72/6000$. Using these p and N-values in the above equation, we obtain $f = 0.50$.

Time-lapse observation in living cells

Time-lapse images of GFP fluorescence in living cells were obtained on the cooled CCD using the computer-controlled fluorescence microscope system (Haraguchi *et al.* 1997). The cells were mounted in a 35-mm glass-bottom culture dish (MatTek

Corp., Ashland, MA) coated with concanavalin A (1 mg/mL) and observed in EMM2 with amino acid supplements at 26 °C using an Olympus oil immersion objective lens (PlanApo 60/NA = 1.4) as previously described (Ding *et al.* 1998).

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