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Gene products required for chromosome separation

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Summary

Gene products required for mitotic chromosome separation in the fission yeast *Schizosaccharomyces pombe* are described. They have been identified by two distinct strategies of mutant isolation, followed by gene cloning and immunochemical characterization of gene products. The roles of four representative genes, namely *nda3*$^+$, *nuc2*$^+$, *top2*$^+$ and *dis2*$^+$, encoding β-tubulin, a nuclear scaffold-like protein, DNA topoisomerase II and type-1 protein phosphatase, respectively, are discussed in regard to the mechanisms and control of chromosome separation.

Introduction

The eukaryotic cell cycle consists of two major events, one duplicating chromosomal DNA in S phase and the other separating the chromatid DNAs into daughter cells in M phase. Each step has to be very accurate, otherwise the progeny cells would receive impaired genetic information. In contrast to a great deal of knowledge accumulated to understand the fidelty of DNA replication, little is known about the regulatory mechanisms that determine how mitotic chromosomes are correctly disjoined.

In mitosis, the chromosomes and cytoplasmic organelles are divided into each of the two daughter cells. The series of the steps leading to chromosome separation are listed below and are similar in most eukaryotes.

(1) Cytoplasmic microtubules disappear.

(2) The nuclear envelope breaks down.

(3) Chromosomes condense, so that individual chromosomes can be seen in prophase.

(4) The elaborate spindle apparatus forms, and the chromosomes display considerable movement.

(5) The chromosomes are aligned onto the metaphase plate.

(6) The sister chromatids of all the chromosomes concertedly disjoin.

(7) The separated chromosomes move toward the poles in anaphase A concomitant with shrinking kinetochore microtubules, followed by spindle elongation, which increases the distance between the poles in anaphase B.

(8) The spindle disappears, leaving a central midbody region.

(9) Chromosomes decondense and the daughter nuclei form.

Even in unicellular lower eukaryotes such as fungi and yeasts, most of these events

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occur (an exception is that the nuclear envelope does not break down in fungi and yeasts), indicating that the mechanisms of motisis are universal. Chromosome separation is also an attractive system in which to study the regulation of higher-order chromosome structures in terms of their dynamic changes in organization during the cell cycle. An intricate motile system involving microtubule shortening and sliding is required for chromosome separation. Understanding mitotic chromosome movement will be a prerequisite to the elucidation of the mechanisms underlying the fidelity of chromosome separation.

We have been investigating chromosome separation by identifying genes and their products required for this process. It seems likely that a large number of genes should be implicated in these events. However, we have focused our efforts on representative genes and have investigated their essential roles in chromosome separation.

The organism we have chosen for our investigation is the fission yeast *Schizosaccharomyces pombe* (Mitchison, 1970). This unicellular eukaryote is convenient for analysis of the cell division cycle and chromosome separation (reviewed by Hirano and Yanagida, 1989). *S. pombe* cells divide by fission (not by budding, as is the case for *Saccharomyces cerevisiae*), and distinct cell cycle stages (G_1, S, G_2 and M) are identifiable. The G_2/M transition is particularly clear. Both haploid and diploid cells are stable. There are three chromosomes per haploid cell (Kohli, 1987; Smith *et al.* 1987; Fan *et al.* 1989). The centromeric DNAs are very large and complex (Nakaseko *et al.* 1986; Chikashige *et al.* 1989). Cell length is maximal just prior to and during M phase. Upon entry into mitosis, cytoplasmic microtubules disappear (Hagan and Hyams, 1988) and a spindle forms in the nucleus (Fig. 1). Actin accumulates at the growing cell ends in interphase (Marks and Hyams, 1985; Marks *et al.* 1986), and during mitosis makes a division ring at the site for cytokinesis. The chromosomes are separated into daughter nuclei by spindle elongation.

The factors required for chromosome separation are classified into *trans*- and *cis*-acting factors. The former are proteins, the gene products, which act on all the chromosomes at a certain mitotic step(s) and are required for chromosome separation. The latter are DNA sequences, in particular regions of the chromosomes that are required for the separation of those chromosomes. The defects in *trans*-acting protein factors (such as tubulin or DNA topoisomerase II) result in the abnormal behaviour of all the chromosomes. On the other hand, the defects in *cis*-acting DNA sequences (such as centromere or telomere) may principally only affect the behaviour of those chromosomes having the defects.

Strategies for identification of the gene products

We have employed two strategies for identifying the *trans*-acting factors (Yanagida *et al.* 1986). Strategy 1 is schematized in Fig. 2. First we isolate *S. pombe* mutants which are temperature-sensitive (*ts*, growth at 26 °C and restrictive at 36 °C) or cold-sensitive (*cs*, growth at 36 °C and restrictive at 20 °C). Following this we employ a secondary screen to select phenotypically interesting mutations. Selection of the

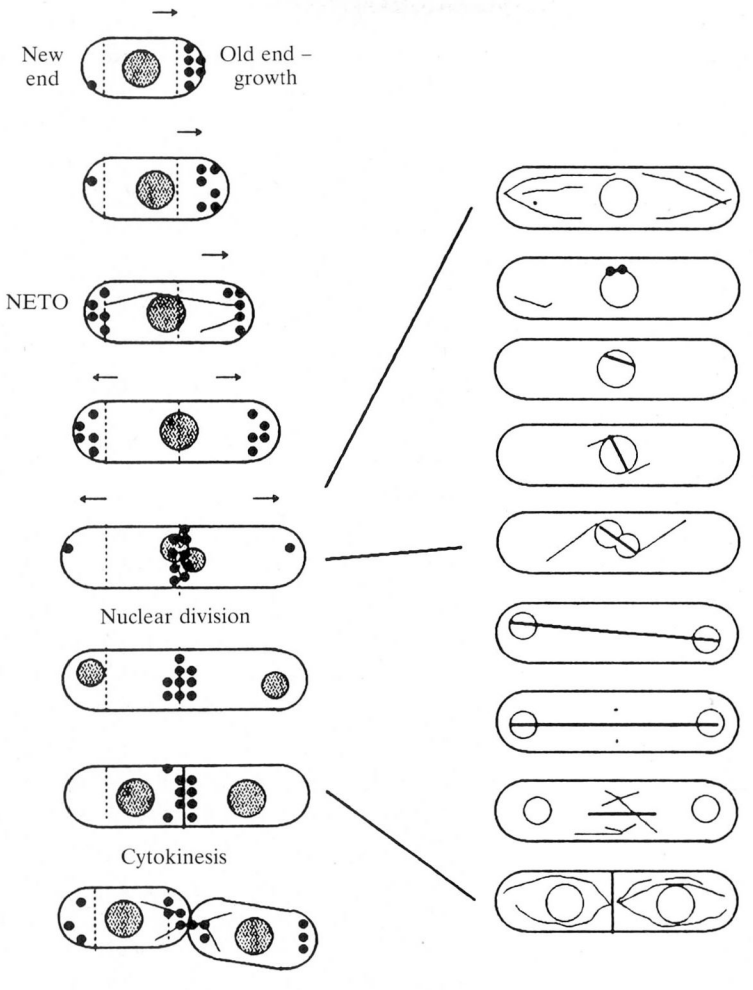

Fig. 1. Cytoskeletal alterations during the cell cycle of *Schizosaccharomyces pombe*. The arrows indicate the direction of cell growth. In the series on the left, dots (also thin filaments) show the distribution of actin, and broken lines represent the division scars. The shaded circles indicate nuclei. In the series on the right, microtubules in mitotic cells are shown by thin filaments and thick lines, respectively (based on Marks *et al.* 1986; Hagan and Hyams, 1988).

mutant phenotype to be looked for in the secondary screen is crucially important, and requires a great number of assumptions. Using this strategy, we have obtained a number of *ts* and *cs* mutants apparently defective in mitotic events, and have identified approximately 50 loci in the genome of *S. pombe* (Hirano and Yanagida, 1989).

The phenotypes of the mutants are characterized in further detail, and the defective mitotic stages are determined. Following this we clone the genomic DNA sequences that complement the *ts* or *cs* phenotype of a mutant by transformation, and determine by chromosome integration whether the cloned DNA is derived from

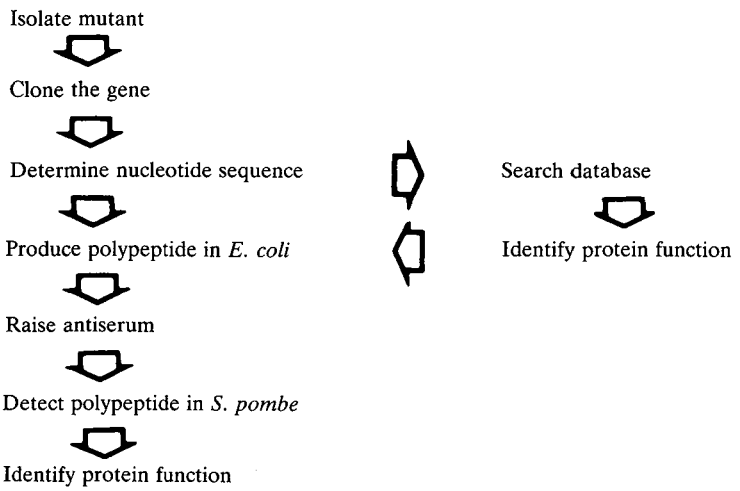

Fig. 2. Strategy 1 to identify gene products required for chromosome separation.

the mutant locus (Rothstein, 1983). Sequencing of the cloned genes (and preferably also the mutant genes) enables us to predict the amino acid sequences of the gene products and search for homology to any known proteins using available databases. As *S. pombe* introns are short and have well-defined consensus sequences (Mertins and Gallwitz, 1987), it is generally possible to predict the amino acid sequences from nucleotide sequences of genomic DNAs. We raised rabbit antibodies against fusion proteins prepared from bacterial cells. Such antibodies recognize the antigens present in extracts of wild-type *S. pombe* strains. It is possible to identify or purify the gene products by immunochemical methods, or to determine their intracellular location in the *S. pombe* cells by immunofluorescence microscopy. Another useful approach to elucidate protein function is to isolate homologous genes from other organisms. If the nucleotide sequence is well conserved in budding yeast or a mammalian organism (Lee and Nurse, 1987), then it seems reasonable to assume that the gene function can also be expected to be conserved. Thus strategy 1 begins by mutant isolation and can be completed by identification of protein function.

The other strategy (2) is to determine whether a known protein activity is required for chromosome separation. Mutants defective in a particular protein (activity) are isolated by assaying directly for the activity in a large number of mutagenized strains. Alternatively, mutants hypersensitive to specific inhibitors are isolated. If the mutants reveal phenotypes defective in chromosome separation, they are further investigated. Mutants of DNA topoisomerases have been isolated by this strategy (Uemura and Yanagida, 1984). Once the genes that complement mutations are isolated, the following steps are similar to strategy 1.

Genes identified by strategies 1 and 2

Some of the genes identified and cloned according to the procedures described above

Table 1. *Fission yeast genes and their products identified by the two strategies*

Genes	No. of amino acid residues in polypeptide	MW (K)	Gene products
nda2$^+$	455	57	α1-tubulin
atb2$^+$	449	55	α2-tubulin
nda3$^+$	448	52	β-tubulin
top1$^+$	812	107	Type I DNA topoisomerase
top2$^+$	1431	160	Type II DNA topoisomerase
nuc1$^+$	1689	190	Largest subunit of RNA polymerase I
nuc2$^+$	665	67	Nuclear scaffold-like protein
cut1$^+$	1827	210	DNA-dependent ATPase
cut2$^+$	301		
dis2$^+$	327	37	Type-1 protein phosphatase
sds21$^+$	322	39	Type-1 protein phosphatase
sds22$^+$	281	30	Leucine-rich repeat nuclear protein
dis3$^+$	970	110	Nuclear protein
crm1$^+$	1077	115	Nuclear and envelope protein

The genes *nda2*$^+$, *atb2*$^+$ and *nda3*$^+$ have been shown to encode α1, α2 and β-tubulin, respectively. DNA topoisomerase I and II genes (*top1*$^+$ and *top2*$^+$, respectively) were identified by strategy 2 (see text). The genes *crm1*$^+$ and *nuc1*$^+$ were identified by their involvement in higher order chromosome structure. Mutations in *crm1* cause drastic alterations in the general architecture of chromosomes and are hypersensitive to Ca^{2+} ions (Adachi and Yanagida, 1989). The *crm1*$^+$ gene encodes a 110K protein which appears to be localized in the nucleus and nuclear periphery. We recently cloned a homologue of *crm1*$^+$ from budding yeast. The mutant *nuc1* shows a phenotype reminiscent of that of a *top1–top2* double mutant. Cloning and sequencing of *nuc1*$^+$ demonstrates that *nuc1*$^+$ encodes the largest subunit of RNA polymerase I (Hirano *et al.* 1989). Both *nuc1* and *top1–top2* destroy nucleolar structure at the restrictive temperature, thus producing similar phenotypes.

in our laboratory are listed in Table 1. Those involved in maintaining chromosome organization are also included. The number of amino acid residues for polypeptides predicted from the determined nucleotide sequences, molecular weights determined by immunoblots of SDS–PAGE using antisera against the fusion proteins, and the nature of the gene products are also shown in the Table. We raised a number of antisera and identified 12 different gene products in *S. pombe* extracts.

Phenotypes of chromosome separation

Phenotypes of representative mutants defective in chromosome separation under restrictive conditions are schematized in Fig. 3. The mutant cells are first exponentially grown at a permissive temperature, then transferred to a non-permissive temperature and incubated for the equivalent of two generations. If a highly uniform arrest phenotype is produced, the mutant cells are presumed to be blocked at a specific stage of the cell cycle. Classes of mutants apparently blocked at distinct stages of mitosis have been isolated. In the *nda3*-311 mutant, the cells are arrested with condensed chromosomes but without the mitotic spindle (Umesono *et al.*

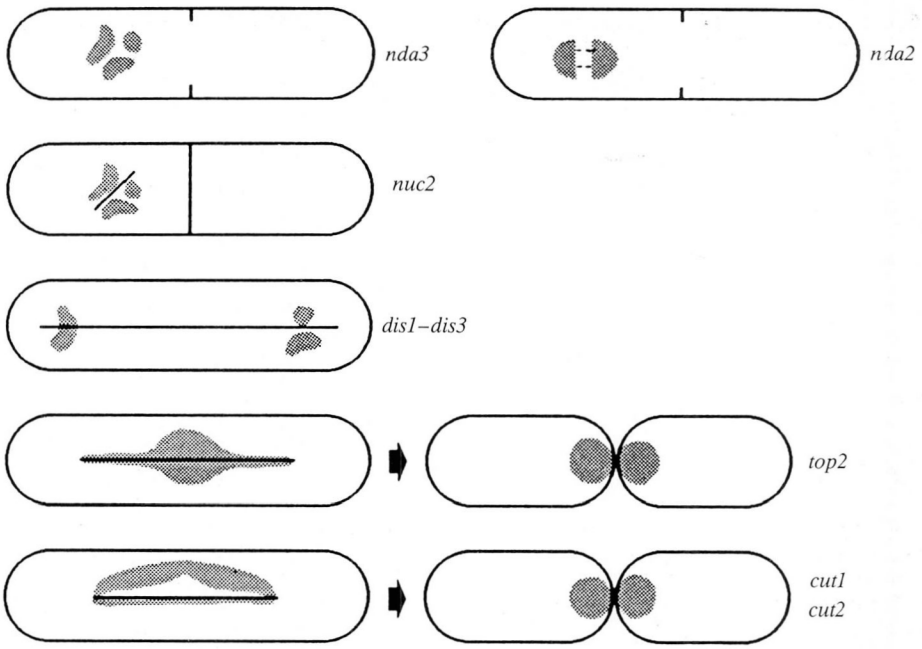

Fig. 3. Phenotypes of mutants defective in chromosome separation at the restrictive temperature. Chromosomes (shaded area) and the spindle (rod) are schematized.

1983*b*). The arrested stage is similar to prophase or prometaphase. Gene cloning and sequencing showed that the *nda3*$^+$ gene encodes β-tubulin (Hiraoka *et al.* 1984).

The phenotype of the *nda2* mutant is similar to *nda3*, but a fraction of the mutant cells at restrictive temperatures proceeds through a metaphase-like stage and are arrested immediately after chromosome separation (Toda *et al.* 1983; Umesono *et al.* 1983*a*). Nucleotide sequencing of the cloned genes that complement *nda2* mutations show that the *nda2*$^+$ gene encodes one of the two α-tubulins (Toda *et al.* 1984). A possible reason for the phenotypic difference between *nda3* and *nda2* mutants is described below.

A mutant called *nuc2* was isolated by its characteristic arrest phenotype with condensed chromosomes at mitotic metaphase; the short uniform-sized spindle is formed but does not elongate (Hirano *et al.* 1988). The nucleus is displaced from the center of cell, as seen in *nda2* and *nda3* cells. The septum is formed but its separation does not occur. The gene product of *nuc2*$^+$ will be described below.

The phenotype of *ts top2* mutants makes a sharp contrast to those described above; most of the mitotic events are not arrested but chromosome separation fails (Uemura and Yanagida, 1984, 1986). In *top2* mutant cells at the restrictive temperature, aberrant chromosomes, which are not fully condensed and are topologically defective, are formed upon entry into mitosis. They are transiently pulled by the spindle, but not successfully separated. Because septum formation and cell separation are not inhibited to *top2* cells the undivided nucleus is bisected by the septum

Table 2. *A summary of the phenotypes of mitotic mutants defective in chromosome separation*

Mutants	Chromosome		Spindle		Septum	Cell separation
	condensation	separation	formation	elongation		
nda2	+	(+)*	(+)*	−	−	−
nda3	+	−	−	−	−	−
nuc2	+	−	+	−	+	−
dis1						
dis2	+	−	+	+	−	−
dis3						
top2	−	−	+	+	+	+
cut1						
cut2	+	−	+	+	+	+
cut10						

*, See text.

References: *nda2*, Toda *et al.* 1983, 1984; *nda3*, Hiraoka *et al.* 1984; *nuc2*, Hirano *et al.* 1988; *dis1–dis3*, Ohkura *et al.* 1988; *top2*, Uemura *et al.* 1987*a*; *cut1* and *cut2*, Hirano *et al.* 1986.

producing two dead daughter cells (this phenotype of uncoupled nuclear division and cell separation is called 'cut'). The mutants *cut1*, *cut2* and *cut10* exhibit phenotypes somewhat similar to that of *top2* (Hirano *et al.* 1986). The phenotypes of these mutants are indistinguishable, showing transiently undivided chromosomes (in a configuration which is termed an 'archery bow'-like structure).

A group of mutants called *dis* produces an intermediate phenotype between *nda3* and *top2*. Three *dis* genes (*dis1*, *dis2* and *dis3*) have been identified so far (Ohkura *et al.* 1988). In these mutants, the spindle is formed and elongates, but chromosomes do not separate. Neither septum formation nor cell separation takes place. Non-disjoined chromosomes are apparently moved to the poles so that they are unequally distributed at the cell ends. The products of the dis^+ genes will be described below.

Thus, uncoupled or uncoordinated mitoses are found in *top2*, *cut*, *dis* and also *nuc2* mutants. Table 2 shows that spindle dynamics, septum formation and/or cell separation can take place in the absence of chromosome separation.

Role of tubulin genes in chromosome separation

The genome of *S. pombe* contains two α- (*nda2* and *atb2*) and one β-tubulin (*nda3*) genes (reviewed by Yanagida, 1987). Therefore in *nda2* mutant cells, a small proportion of functional microtubules (consisting of $atb2^+$ and $nda3^+$ tubulin) may be formed even at the restrictive temperature (Adachi *et al.* 1986). This may explain the difference in the phenotypes of *nda2* and *nda3* mutations. In the *nda3* mutant, functional microtubules would be completely absent, so that the spindle does not form, causing the arrest at a stage similar to prometaphase. In *nda2*, an incomplete spindle might be formed, which is able to separate chromosomes but unable to increase the pole to pole distance. As many long microtubules might be required for

anaphase B spindle elongation, anaphase A and B would be distinct in regard to their dosage requirements for functional microtubules.

One *cs* allele *nda3*-311 is useful for analysis of mitotic events, as the mutant β-tubulin is reversibly inactivated (Hiraoka *et al.* 1984). At restrictive temperatures, the cells are uniformly arrested but upon transfer to the permissive temperature, the cells proceed highly synchronously into anaphase; thus cellular β-tubulin is rapidly reactivated and the spindle is immediately formed, followed by a highly concerted chromosome separation. The rate of spindle elongation is $1\,\mu m\,sec^{-1}$, roughly half that of wild-type spindle elongation (Tanaka and Kanbe, 1986; Hagan, I. and Hyams, J., personal communication).

Requirement of topoII for chromosome condensation and separation

We isolated the ts *top1* and *top2* mutants by strategy 2 (Uemura and Yanagida, 1984). The *top2* mutants contain a heat-sensitive topoII enzyme irreversibly inactivated at 37°C. A *cs top2* mutant which contained the topoII activity reversibly inactivated at 20°C was also isolated (Uemura *et al.* 1987*a*). Gene disruption experiments indicate that the *top2* gene is essential, whereas the *top1* gene is dispensable (Uemura *et al.* 1987*b*; Shiozaki, K. and Yanagida, M., unpublished). The defect of *top1* can be substituted by a sufficient amount of topoII.

The *ts top2* mutants at the restrictive temperature produce abnormal chromosomes at mitosis; these are transiently extended into filamentous structures along the elongating mitotic spindle, but are not separated (Uemura and Yanagida, 1986). A primary defect in *top2* appears to be the formation of aberrant mitotic chromosomes inseparable by the force generated by the spindle apparatus.

Reciprocal temperature-shift experiments using the double mutants of either *ts* or *cs top2* and *cs nda3* indicated that topoII is required for chromosome condensation and separation (Uemura *et al.* 1987*a*). A *cs top2-cs–nda3* double mutant at 20°C shows long, entangled chromosomes, which condense and separate upon shift to the permissive temperature. If spindle formation is prevented at the permissive temperature, the chromosomes condense but do not separate. Thus topoII appears to be required for final chromosome condensation. Moreover pulse-shift experiments show that topoII is required for chromatid disjunction. Experiments with *ts top2-cs–nda3* cells show that topoII is also required for chromosome separation in anaphase: inactivation of topoII and activation of β-tubulin allow normal spindle formation but result in 'streaked' chromosomes. The topoII activity of decatenation/catenation and knotting/unknotting of DNA appears to become essential during mitosis. DNA relaxing activity is abundant in the *top2* mutant cells due to the presence of topoI.

In yeasts, topoII is not essential for replication and transcription if the amount of topoI is sufficiently high (reviewed by Yanagida and Wang, 1987). If either topoI or topoII is absent, replication and rRNA synthesis proceeds normally. Studies of the double mutant *top1–top2* indicate that DNA relaxing activity is essential for replication, rRNA transcription and nucleolar organization. The effect of combining

ts nucl (defective in the largest subunit of RNA polymerase I) and *ts top1–top2* shows that the topoI and II enzymes are required for folding of rDNA and RNA polymerase I molecules into the assembly of nucleolar genes to allow their function (Hirano *et al.* 1989).

The amino acid sequence of topoII, deduced from the cloned *top2*$^+$ gene, reveals homology to prokaryotic type II DNA topoisomerase II, gyrase (Uemura *et al.* 1986). The NH_2 terminal domain of topoII is similar to the ATP binding B-subunit of gyrase, the central COOH region resembles the DNA binding A-subunit and the COOH terminal domain consists of highly charged residues. The topoII sequences of other organisms such as budding yeast and human also show these three domains.

We are dissecting essential and non-essential domains in topoII to try to understand the role of the enzyme in mitosis. The mechanism of mitotic activation of the enzyme is particularly interesting. For this purpose, we are attempting to identify an alteration in the enzyme structure required for its mitotic activity. A possible one is phosphorylation and dephosphorylation of topo II.

Role of *cut*$^+$ genes and their products

The phenotypes of *cut1*, *cut2* and *cut10* mutants are similar, suggesting that their gene functions are related (Hirano *et al.* 1986; Uzawa, S., unpublished results). Consistently, the cloned *cut1*$^+$ gene is able to complement not only *cut1* but also *cut2* and *cut10* mutants. The cloned *cut2*$^+$ and *cut10*$^+$ genes, however, do not complement *cut1* mutants. The product of the *cut1*$^+$ gene has recently been identified (Uzawa, S. *et al.* to be published). It is a minor nuclear protein of 210K ($K = 10^3 M_r$) containing a consensus sequence for ATPase. The nucleotide sequence suggests that the *cut1*$^+$ gene product is a novel DNA-dependent ATPase, essential for chromosome separation.

Role of the *nuc2* gene as a nuclear component

Mutant *ts nuc2* cells enter mitosis with normal timing under restrictive conditions, and are arrested at a metaphase-like stage (Hirano *et al.* 1988). The chromosomes are condensed but do not separate. A short uniform-sized spindle forms but does not elongate (Fig. 4). The chromosomes are arranged so that a plate-like structure is formed through the center of which the spindle runs, perpendicular to the plate. It is important to determine whether chromosome structures or the spindle is defective in the *nuc2* mutant.

The nucleotide sequence of the cloned *nuc2*$^+$ gene predicts a 76K protein with several internal repeats (Hirano *et al.* 1988). Gene disruption indicates that the *nuc2*$^+$ gene is essential. To identify the *nuc2*$^+$ gene product, antisera against fusion proteins were made. Immunoblotting detects a polypeptide (apparent MW 67K, designated p67) in wild-type extracts of *S. pombe*. (In extracts of the mutant prepared after incubation at the restrictive temperature, a polypeptide (MW 76K) in addition to p67 is found.) The amount of p67 in wild-type extracts is greatly increased by introduction of multicopy plasmids carrying the *nuc2*$^+$ gene. Plasmids

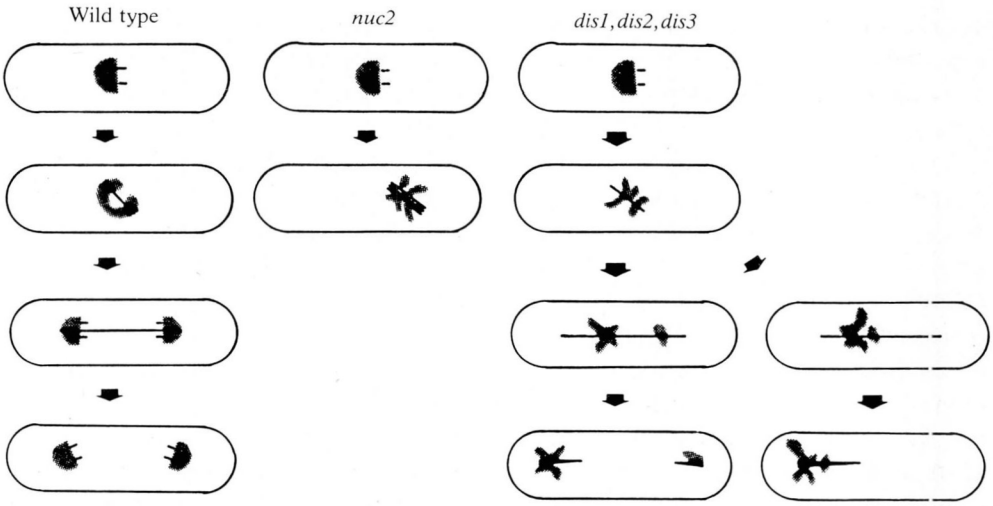

Fig. 4. Defective phenotypes of *nuc2* and *dis* mutants at the restrictive temperature. See text.

carrying the $nuc2^+$ gene with a NH_2 domain deleted produce a shorter polypeptide with the expected MW, therefore, the bands detected by immunoblotting should represents the $nuc2^+$ gene product. To determine its cellular location, homogenates were run in a Percoll gradient and each fraction was analyzed by immunoblotting. Results indicate that most of the p67 is present in nuclear fractions. (Immunofluorescence microscopy has failed to localize the $nuc2^+$ protein in wild-type cells.)

p67 is an insoluble nuclear protein, and behaves as a nuclear scaffold-like protein (Hirano *et al.* 1988). It is insoluble in 2 M-NaCl, 25 mM-LIS and 2% Triton but is soluble in 8 M-urea. The p76 protein found in mutant extracts, however, is soluble. Recently we found that the *nuc2* protein made in *Escherichia coli* binds tightly to DNA (Hirano, T., unpublished result). This DNA binding activity is localized to a small region of the polypeptide. Furthermore, weak but significant homology was found to certain gene products of *S. cerevisiae* related to mitosis and the induction of gene expression (R. S. Sikorski, M. Goebl, M. Boguski and P. Hieter, personal communication). Therefore, it is most likely that the $nuc2^+$-related proteins are universally present in eukaryotes, and constitute a new class of gene family. A potential molecular function of *nuc2* and related genes would be in altering chromosome structures by interacting simultaneously with chromosome DNA and the nuclear scaffold. It is of great interest to see whether the *nuc2* protein associates with specific sites in the chromosomes.

Isolation of *dis* mutants and their phenotype

The *dis* mutants were isolated by screening approximately 1000 *cs* strains. They show basically the same cytological phenotype, but are classified into three complementation groups, designated *dis1*, *dis2* and *dis3* (Ohkura *et al.* 1988). The

dis mutant cells were first grown exponentially at permissive temperature and separated in a sucrose gradient by centrifugation to select early G_2 phase cells. Then, the synchronized cells were incubated at the restrictive temperature. The *dis* mutant cells became lethal during mitosis, suggesting that the dis^+ genes are essential in mitosis. The terminal phenotype shows the asymmetric distribution (3:0 or 2:1) of the nondisjoined, condensed chromosomes at the two ends of cell. If the above cells are incubated at permissive temperature, mitotic chromosome separation proceeds normally. The *dis* mutants are not defective in DNA replication.

The phenotypes of the *dis* mutants are pleiotropic, as listed below (Ohkura *et al.* 1988).

(1) Chromosomes strongly condense under restrictive conditions. The extent of condensation appears to be much greater than that of the wild type.

(2) The early spindle structures seen in the cells at the restrictive temperature lack the thick ends normally seen in the wild-type early spindle structure and considered to be kinetochore microtubules (Hiraoka *et al.* 1984; Hagan and Hyams, 1988). Thus *dis* mutants may be impaired in kinetochore structure.

(3) The intermediary segregating form (called the U-form) of chromosomes seen in the wild type (Toda *et al.* 1981) is never seen in *dis* mutant cells. Instead the chromosomal domain appears to be split into three subdomains (presumably corresponding to the three chromosomes) after the spindle forms. Therefore sister chromatid separation is defective in the *dis* mutants.

(4) Non-disjoined chromosomes move to the cell ends with either a 2:1 or 3:0 distribution.

(5) The nucleolus does not separate.

(6) Terminal degradation of the mitotic spindle is not complete. The parts associated with the condensed chromosomes remain.

(7) Decondensation of chromosomes does not occur.

(8) Artificial minichromosomes are lost with a high frequency at the permissive temperature.

(9) Homozygous diploids cannot be made at any temperature.

(10) All of the isolated *dis* mutants are hypersensitive to caffeine. Mutant cells in the presence of caffeine at permissive temperature show a phenotype similar to that expressed at non-permissive temperature.

These phenotypes indicate that the dis^+ genes play a fundamental role in chromosome behaviour. Genes that complement the *cs dis* phenotype have been cloned by complementation (Ohkura *et al.* 1988). Interestingly, there are six clones that complement *dis1* mutants while four clones complement *dis2*. Only one clone complements *dis3*. There appears to be a complex interacting system to execute the dis^+ gene functions.

Genes that complement *dis2* and their products

One of the four genomic sequences that complements *dis2*-11 directs integration to a chromosomal site which is tightly linked to the *dis2* locus, suggesting that it contains

the *dis2*$^+$ gene. The other three genes are not linked to *dis2*, and are designated *sds21*$^+$, *sds22*$^+$ and *sds23*$^+$. Interestingly, the *dis2*$^+$ and *sds21*$^+$ genes complement both *cs* and caffeine hypersensitivity, whereas the other two complement only the *cs* phenotype (Ohkura *et al.* 1989).

By nucleotide sequence determination, the *dis2*$^+$ and *sds21*$^+$ genes are found to encode highly homologous proteins (calculated size approximately 37K). Antiserum raised against the fused *dis2*$^+$ detects two polypeptides of 37K and 39K in the extracts of wild-type *S. pombe*. The intensity of the major p37 band was increased by the presence of a multicopy plasmid carrying the *dis2*$^+$ gene, while the minor p39 band increases its intensity by a multicopy plasmid carrying the *sds21*$^+$ gene. Thus we conclude that *dis2*$^+$ and *sds21*$^+$ genes encode p37 and p39, respectively. By immunochemical analyses, p37 and p39 are found to be preferentially enriched in the nucleus; they exist as oligomers and are solubilized by 0.4 M-NaCl. Immunofluorescence microscopy shows that the nucleus is intensely stained, and granules are seen in the cytoplasm.

The *dis3*$^+$ gene was cloned by complementation. By nucleotide sequence determination and immunochemical analyses, we found that it encodes a protein whose behaviour is highly similar to that of the *dis2*$^+$ protein (Kinoshita, N., unpublished result).

Genes similar to *dis2*$^+$ have been cloned from other organisms. By hybridization, the sequences highly homologous to *dis2*$^+$ were obtained from *S. cerevisiae* and mouse (Ohkura *et al.* 1989). The predicted amino acid sequences of the budding yeast and mouse clones are more than 80 % identical to that of *dis2*$^+$. Thus the *dis2*$^+$ gene, and concomitantly its function, is highly conserved from yeasts to mammals. It was found that the *dis2*$^+$ protein is highly homologous to a rabbit protein phosphatase 1 (Ohkura *et al.* 1989). The significance of this finding is described in the following section.

The *sds22*$^+$ gene has been cloned, and its nucleotide sequence shows that the *sds22*$^+$ gene product consists of a series of leucine-rich repeats (Ohkura, H., unpublished result). Characterization of this protein by immunoblotting using antiserum raised against a *sds22*$^+$ fusion protein indicates that this leucine-rich protein exists in the nucleus.

Roles of *trans*-acting protein factors in chromosome separation

Our results are summarized in Fig. 5. These gene products are essential for chromosome separation. Essentiallity of the genes can be examined only through genetical investigation. These may represent only a small fraction of the genes required for chromosome separation.

It is still a surprise that tubulin mutants show cell cycle arrest phenotypes, considering that microtubules are involved in a wide range of cellular functions. Our results indicate that certain tubulin gene mutations cause specific arrest at a mitotic stage by the inability of the cells to form a mitotic spindle. There may be other cell cycle-associated genes that act on the mitotic spindle. Their mutant phenotypes

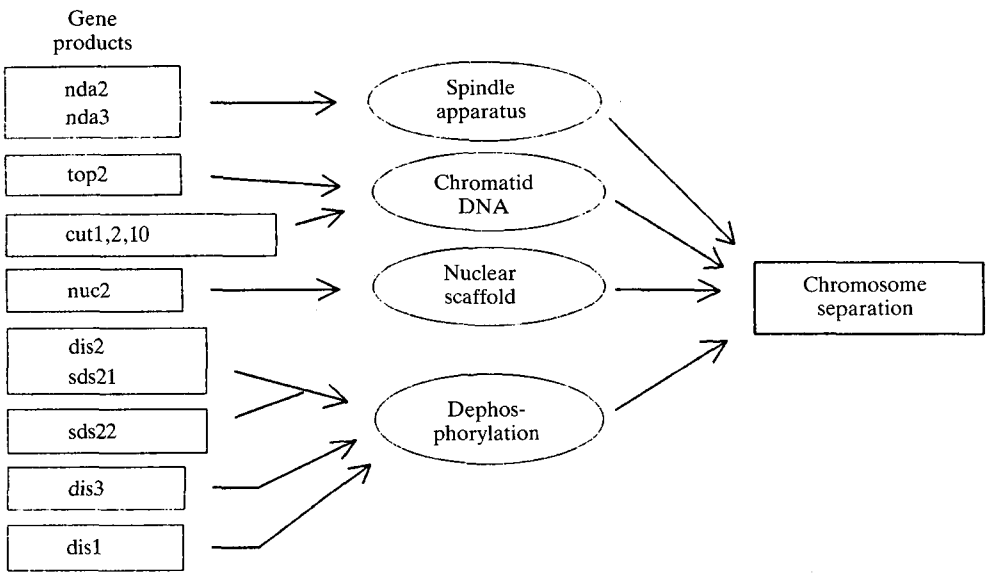

Fig. 5. The roles of gene products required for chromosome separation in *S. pombe*. See text.

would possibly be expected to be similar to those of tubulin mutants. Therefore, we have isolated such mutants and are characterizing them, hoping that some of them are related to microtubule-associating or interacting proteins.

There may also be DNA-interacting enzymes present other than topoII that are required for condensation and segregation of sister chromatids. An example would be the *cut1*$^+$ protein. The *cut1* mutants exhibit phenotypes similar to those of *top2* mutants, and the *cut1*$^+$ protein seems to encode a 210K potential DNA-dependent ATPase. Other mutations involved in the covalent bond changes in mitotic chromosome DNAs may show similar phenotypes. The defects caused by *top2* and *cut* are perhaps not recognized by the regulatory systems for spindle dynamics and cytokinesis.

The nuclear scaffold-like protein nuc2$^+$ is required for chromosome separation. It is unknown why the *nuc2* mutant is blocked at a stage similar to metaphase. A hypothesis is that the *nuc2*$^+$ gene product is required for a gross structural alteration of chromosome structures during the metaphase–anaphase transition. It will be interesting to see whether the nuc2 protein interacts with specific sequences of DNA, or nuclear scaffold, or both. Further investigation is necessary to understand the role of the nuc2$^+$ protein, especially with regard to its subdomain and repeat units.

The *dis2*$^+$ protein is highly homolgous to rabbit protein phosphatase-1, and therefore the *dis* phenotype may be related to dephosphorylation. Approximately 82% and 74% of the residues of *dis2*$^+$ and *sds21*$^+$ proteins, respectively, are identical to rabbit protein phosphatase 1 (PP-1; Berndt *et al.* 1987; Cohen, 1988). These values of similarity are very high, considering the evolutionary distance

between yeast and rabbit. The gene disruption experiments indicate that single disruptants are viable, where the double disruption is lethal; *dis2*$^+$ and *sds21*$^+$ are functionally overlapped, although they may not be identical. The disruption of *dis2*$^+$ may be substituted by *sds21*$^+$ and *vice versa*.

The defect in protein phosphatases may well cause a much more general phenotype in cellular function as the enzymes are involved in many cellular processes. However, the arrested phenotype of *dis2* is apparently highly uniform and specific for mitosis. Genes similar to PP-1 were also isolated from *S. pombe* and *Aspergillus nidulans* using cell cycle-specific phenotypes. The gene *bws1*$^+$ of *S. pombe*, which reverses *wee1* mutant suppression of the *cdc25* mutation, is identical to the *dis2*$^+$ gene (Booher and Beach, 1989). The phenotype of *A. nidulans bimG* mutant is similar to that of the *dis* mutants, and the gene complementing *bimG* is highly homologous to PP-1 (Doonan and Morris, 1989). Therefore the role of PP-1 may be specifically implicated in mitotic control. It has been pointed out that protein phosphatases would be expected to play an important role in mitosis (Foulkers and Maller, 1982; Cyert and Kirschner, 1988). However, only four types of protein phosphatases have been described in mammalian systems (Ingebritsen and Cohen, 1983). This is in contrast to the large number of protein kinases in which mutations often cause a cell cycle stage-specific phenotype. There may be many more protein phosphatases awaiting discovery or there may be some unknown mechanisms which attribute a wide variety of substrate specificities to a restricted number of protein phosphatases.

How does a mutation in the structural gene for protein phosphatase 1 cause the block of chromosome disjoining? Current hypotheses are: (1) alteration in the dephosphorylation pattern causes hypercondensation of chromosomes which are unable to be disjoined. In this case, mitotic induction is too strong to be properly regulated; (2) coordinate coupling of PP-1 with mitotic kinases is broken, so that chromosome separation is blocked while chromosomes condense and the spindle is made and elongates, and (3) temporally-regulated expression of type 1 protein phosphatase is essential for correct segregation of chromosomes so that mutations impaired in such temporal control results in the *dis* phenotype.

The *dis2*$^+$ might be implicated in the cAMP cascade pathway because *dis2*-11^{cs} is weakly complemented by the *S. cerevisiae PDE2* (cAMP phosphodiesterase gene; Ohkura *et al.* 1988). The mutation can be rescued by apparently decreasing the intracellular cAMP concentration. It is clear that the expression of *dis2*$^+$/PP-1 is under the control of a highly complex interacting system.

In conclusion, we have identified some of the gene products required for mitotic chromosome separation in the fission yeast *S. pombe* by mutation, gene cloning, nucleotide sequence and immunological methods. They include; α-, β-tubulin, DNA topoisomerase II, a nuclear scaffold-like protein nuc2$^+$ and type 1 protein phosphatase. Interestingly, none of them apparently has a unique role in mitosis, but instead, they have multiple cellular roles. However, mutations which cause defects at specific steps in chromosome separation can be identified in these genes. This apparent contradiction may be understood by the fact that chromosome separation is

a highly intricate and complex system that involves the coordinated function of a number of inter-related gene products. For example, *top2* mutations specifically block chromosome condensation and separation, because functionally overlapping topo I can substitute most of the topo II functions in other steps of the cell cycle. A topo II function which cannot be substituted by any other gene product becomes essential during chromosome separation. Certain gene products have to be activated, modulated or inactivated during mitosis with correct temporal control. Therefore the study of chromosome separation requires an understanding of many gene functions involved in cell growth control, transcription or DNA replication. Examples are shown in this paper. Mutations affecting such mitosis-specific modulations will cause a defect in chromosome separation. Genetic analyses of chromosome separation have revealed a number of regulatory mechanisms, the coordinate execution of which can be uncoupled. A major conclusion from our studies to date is that there are at least four potentially independent pathways in mitosis in the fission yeast, namely chromosome separation, spindle dynamics, septum formation and cytokinesis.

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