

MOLECULAR GENETICS OF THE cdc 22 GENE OF
SCHIZOSACCHAROMYCES POMBE.

by

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Thesis presented for the degree of Doctor of Philosophy of
the University of Edinburgh in the Faculty of Science.

1985



Regulation 2.4.15

I declare that this thesis was composed by myself, and that the research presented is my own work. Due acknowledgement is made within the text for contributions from other sources.

For my family.

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Abbreviations

BSA	bovine serum albumin
<u>cdc</u>	cell division cycle
DNA	deoxyribonucleic acid
EMM	Edinburgh minimal medium
EDTA	ethylene diamine tetra-acetic acid
Kb	kilobase
ME	malt extract
NDPK	nucleoside diphosphokinase
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
TNS	tri-iso-propyl naphthalene sulphonic acid
Tris	tris (hydroxymethyl) methylamine
UV	ultra violet
YE	yeast extract
YEPD	yeast extract peptone dextrose

ABSTRACT OF THESIS

The cloning procedure used for the isolation of the cdc 22 gene was to transform a cdc 22 - M45 strain with a pool of wild type S. pombe DNA fragments carried in a plasmid gene library and screen for complementation of the mutation at the restrictive temperature. Eight separate yeast cdc⁺ transformants were isolated by this method.

The complementing plasmids were purified and subjected to restriction digest analysis. This showed that either of two independent sequences of 5.6 Kb and 2.2 Kb in size could mediate rescue of the mutation. In addition genomic digests followed by Southern blot analysis, using each cloned sequence as a hybridisation probe, showed that neither sequence was the result of a ligation artifact formed during the construction of the gene library and that each was present as a single copy in the S. pombe genome.

To determine which, if any, of the two cloned DNA fragments contained the cdc 22⁺ gene both sequences were integrated by homology into the S. pombe genome. The site of integration was then mapped genetically relative to the cdc 22 locus. The data obtained showed that the site of integration of the 5.6 Kb fragment was tightly linked to the cdc 22 locus. In addition all the integrants were cdc⁺ at the restrictive temperature. However, the site of integration of the 2.2 Kb fragment was unlinked to the cdc 22 locus, and all the integrants were cdc⁻. Therefore, it was concluded that the cdc 22⁺ gene was present within the 5.6 Kb fragment and that the 2.2 Kb fragment contained an extragenic suppressor that only suppressed when present at a high copy number.

The cloned genes were then used as hybridisation probes in Northern blot analysis of wild type RNA. Using the 5.6 Kb fragment as a probe a single homologous transcript of 3.3 Kb in size was detected. To determine if the appearance of the cdc 22 mRNA was dependent on the completion of a specific cell cycle event RNA was made from six different cdc⁻ mutant strains that arrested at different points in the cell cycle.

The RNA obtained was subjected to Northern analysis using the 5.6 Kb fragment as a probe. The results obtained showed that the cdc 22 transcript was present in cells blocked at each stage in the cell cycle. Therefore the appearance of the cdc 22 mRNA was not dependent on the completion of a specific cell cycle event. In order to investigate the level of cdc 22 transcript through the cell cycle RNA samples were prepared from a synchronous culture obtained by selection from an elutriator rotor. The RNA samples were probed with the cloned cdc 22 DNA by Northern analysis. These experiments clearly demonstrated that the cdc 22 mRNA was cell cycle regulated reaching a maximum level during late G₁ of the cell cycle.

Similar experiments were carried out using the cloned extragenic suppressor as a hybridisation probe. When total RNA from wild type cells grown at 25°C was probed with the 2.2 Kb fragment by Northern analysis a single transcript of 1.5 Kb in size was detected. However, when the cell cycle dependency of the accumulation of homologous transcripts was investigated by using the same six cdc mutant strains to block cells at different points in the cell cycle an interesting transcript pattern was observed. With RNA obtained from five out of six of the blocked mutant strains an additional transcript of 2.4 Kb in size could be detected. However this larger transcript was absent from the remaining cdc⁻ mutant strain and also from wild type cells grown at the restrictive temperature. The absence of the larger transcript in wild type cells grown at the restrictive temperature shows that its appearance cannot be the result of a heat shock response due to incubation at the higher temperature. When the suppressor DNA was used to probe RNA obtained from synchronous cultures of wild type cells, only the 1.5 Kb transcript was easily detected. In contrast to the cdc 22 transcript no cell cycle fluctuations in the level of the 1.5 Kb transcript was observed.

CHAPTER 1

1 Introduction

The cell cycle is the sequence of events that takes place between the birth of a cell and its division into two daughters. During this time, if growth is "balanced", the mother cell doubles all of its cellular constituents.

Up to the early 1950s, cell biologists had defined the eukaryotic cell cycle as composed of two processes observed by light microscopy, interphase and mitosis. Typically, interphase was found to occupy 95% of the cycle during which no visible changes could be observed. Interphase was thought to be the time in which cell growth and preparations for division occurred. This was followed by mitosis when the chromosomes condensed and separated to the two daughter cells. As no biochemical methods were available for the study of cell cycle events during interphase, most early work up to this time focussed on the dramatic events that occurred during mitosis which could be studied by direct microscopic observation.

However, in the early 1950s, biochemical techniques such as autoradiography that could be used for studying macromolecular synthesis were developed. The use of these new methods depended on sufficient biological material being available to carry out the measurements. This problem was solved by the introduction of a number of different approaches to obtain synchronous populations of a wide range of cells (See next section). Biochemical analysis showed that most cell components, for example, total protein and total RNA, were synthesised continuously throughout interphase. One important exception in eukaryotic cells was DNA synthesis, which was shown by Howard and

Pelc (1953) to take place during a restricted period of interphase. This observation was used to subdivide interphase into three intervals. The period of DNA synthesis was termed S phase. The time between the previous mitosis and the start of DNA synthesis was termed G_1 , and the remaining period between the completion of DNA synthesis and next mitosis, G_2 .

Due to the importance of synchronous cultures in studying macromolecular synthesis the various methods used are discussed in the next section. This is followed by a review of the accumulation of proteins through the cell cycle using synchronous cultures of a number of different organisms.

2 Synchronous Cultures

To measure individual enzyme activities or protein levels on single cells is technically difficult. Therefore, a major problem in the study of individual protein levels through the cell cycle, is to obtain enough material for biochemical assays at each stage of the cell cycle. A solution to this difficulty is to prepare a synchronously dividing population of cells which, when sampled at different points in the cell cycle, provide sufficient material for biochemical analysis. One approach is to investigate cell populations which divide with a natural synchrony such as cleavage in fertilised eggs (Mazia and Dan, 1952). Another useful system has been the synchronous mitotic division of nuclei found in the plasmodium form of the slime mould Physarum polycephalum (Rusch, 1969). An alternative and more widely used approach is to study cell populations in which synchrony has been artificially obtained. Two procedures termed induction and selection synchrony are used to obtain experimentally derived synchronous cultures.

In induction synchrony, a synchronous population is achieved by blocking and then releasing cells at a particular point in the cell cycle. This may be conferred by an environmental stimulus such as light, temperature or nutrient limitation. For example, highly synchronous populations of Chlorella have been obtained by repeated exposure to alternate periods of light and darkness of 15 and 9 hours respectively (Tamiga et al, 1953; Atkinson et al, 1974).

A different induction procedure involves the use of a chemical inhibitor to block cells. Inhibitors are available which specifically prevent completion of either DNA synthesis or mitosis (Mitchison, 1971). On removal of the inhibitor the population divides synchronously.

One advantage of induction synchrony procedures is that, in principle, all the cells in the original asynchronous culture are used to prepare the synchronous culture. An important disadvantage with induction techniques is that they have been shown to cause cell cycle perturbations in a number of systems (discussed in Mitchison, 1971).

The second major approach used to prepare synchronous cultures is by selection. Cells at a particular point in the cell cycle are selected from an asynchronous population and cultured separately as a synchronous culture. Several methods of selection are available and four of the most commonly used are described in the following section.

A selection method commonly used in the synchronisation of mammalian cells is wash off or selective detachment (Terasima and Tolmash, 1963). The principle behind the technique is that cultured mammalian cells round up and adhere less well to the culture dish during the latter stages of mitosis. Mitotic cells may be collected following gentle washing of the monolayer and the selected cells grown as a synchronous culture.

Another analogous selection technique called membrane elution has been used to synchronise cells of Escherichia coli (Helmsetter and Cummins, 1963). This relies on the specific ability of the E. coli strain B/r to bind firmly to nitrocellulose filters. From the mother cells bound to the filter surface, daughter cells are released into circulating growth medium. These daughter cells are collected and grown up as a synchronous culture.

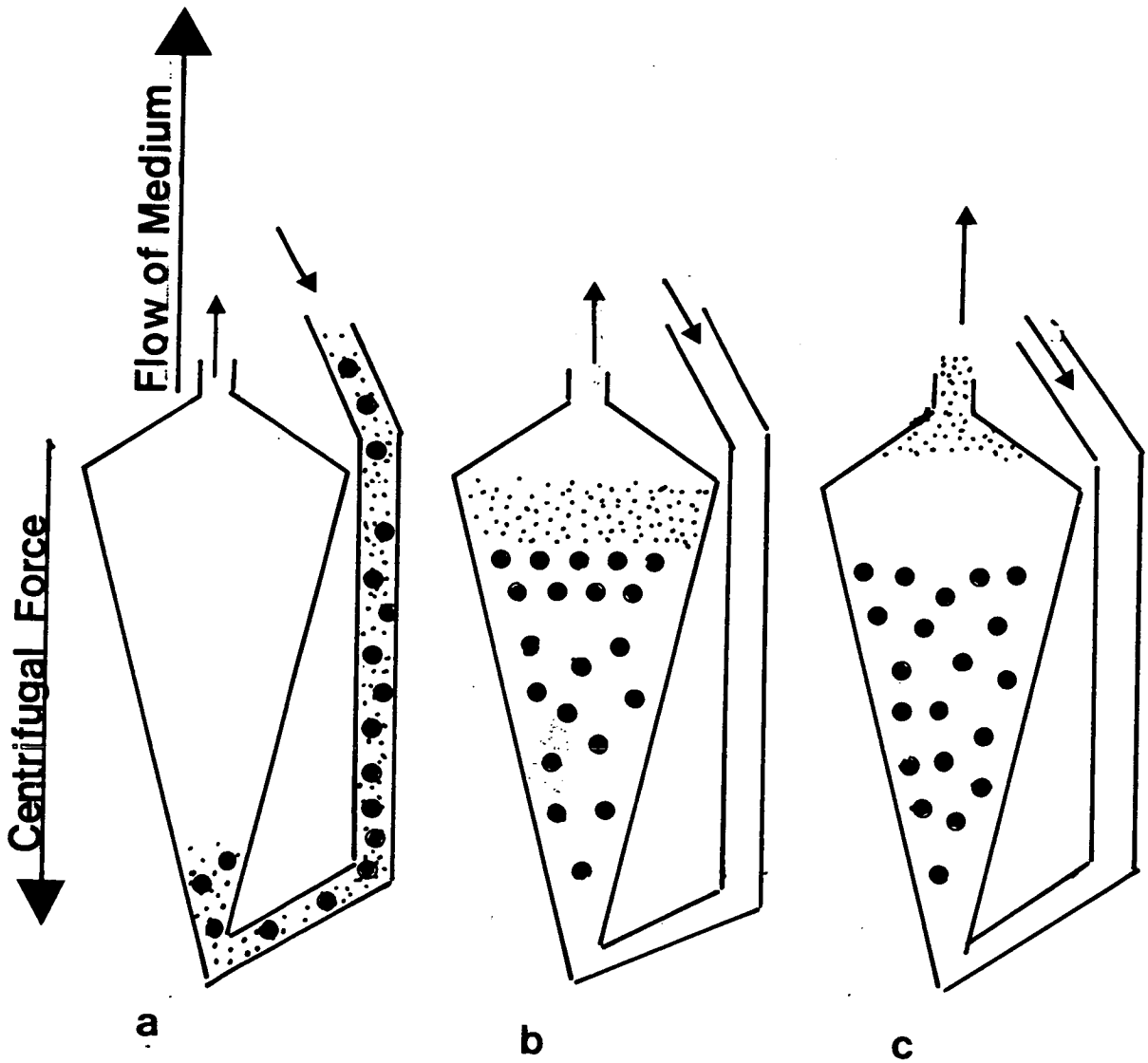
A popular method, especially for obtaining synchronous cultures of bacteria and yeast, is the selection of small cells from a sucrose gradient. In this method originally developed by Mitchison and Vincent (1965), cells from an asynchronous population are collected

resuspended in a small volume of growth medium and layered on top of a sucrose gradient. Following centrifugation the cells are distributed through the gradient according to size, with smaller cells at the top and larger cells at the bottom. A fraction containing small cells is harvested from the top of the gradient and resuspended in fresh medium to obtain a synchronous culture.

A more recently developed selection technique for mammalian cells and yeast involves the selection of cells from an elutriating rotor (Meistrich et al, 1977; Gordon and Elliot, 1977; Creanor and Mitchison, 1979). The elutriator rotor works by a counter current method. In simple terms, centrifugation generates an outward force against cells in a 4 ml rotor sample cell which is balanced by an inward flow of medium. The result is that cells are kept suspended in the rotor sample cell. Furthermore these cells in the rotor sample cell are separated according to size with small cells at the top, and large cells at the bottom. By increasing the pump rate of the medium through the rotor small cells at the top of the rotor cell can be harvested to prepare a synchronous culture. A diagram explaining the principles of cell separation on an elutriating rotor is shown in figure 1.1.

It is generally accepted that methods used to obtain selection synchrony produce less physiological perturbation of cells than induction methods (discussed in Mitchison, 1971). Nevertheless some selection methods are also considered to produce metabolic perturbations when used to prepare synchronous cultures (Mitchison, 1977; this topic is covered further in the next section). For instance, Creanor and Mitchison (1979) have convincingly demonstrated that synchronous cultures of fission yeast prepared by elutriation show

Figure 1.1 Principle of Cell Separation by an Elutriating Rotor



Cells (●,•), suspended in growth medium, are pumped into the rotor chamber (Figure 1.1a). The centrifugal force is balanced by the inward flow of medium. The cells are separated according to size in the rotor chamber, small cells (•) at the top and large cells (●) at the bottom (Figure 1.1b). The small cells are harvested by increasing the flow rate (Figure 1.1c).

less perturbations than those obtained by selection from a sucrose gradient.

The main disadvantage of selection methods is that cell yields are low since only a proportion of the original asynchronous population is used to prepare the synchronous culture. A modification to improve the yield with cells separated on an elutriator rotor or sucrose gradient is to "age fractionate" the culture (Gordon and Elliot, 1977; Sebastian et al, 1971). This technique exploits the fact that cells are separated according to size and thus cell cycle position down the gradient or rotor. By collecting successive fractions, cells at all stages of the cell cycle are obtained. Biochemical assays are carried out directly on the cells obtained from these fractions.

Age fractionation has a number of disadvantages. For example, cells cannot be followed through successive cycles and in practice it has proved difficult to resolve cells obtained from specific fractions into discrete stages of the cell cycle (Mitchison, 1971; Creanor et al, 1983).

3 Accumulation of proteins during the cell cycle

Studies on the increase in total protein content showed that protein accumulated continuously throughout interphase (Mitchison, 1971). It was therefore surprising that when the activities of a number of different enzymes were measured in synchronous cultures of bacteria and yeast, most exhibited a periodic increase or "step" in activity at a particular time in the cell cycle (Gorman et al, 1964; Masters et al, 1964). In these experiments, it was assumed that

enzyme activity was directly related to the amount of enzyme present.

This raised the interesting dilemma that most enzymes were periodically synthesised while total protein accumulated continuously through interphase. To explain these seemingly contrasting observations it was postulated that the continuous rise in total protein resulted from the sum of individual proteins each being synthesised periodically as "steps" at different times in the cell cycle (Gorman et al, 1964).

A large body of similar work followed, related to the increases in enzyme activity using synchronous cultures of a number of different organisms such as Chlorella, Physarum, mammalian cells, bacteria, budding and fission yeast (reviewed by Mitchison 1971; 1977). The increases in enzyme activities through the cell cycle was studied most extensively in the budding yeast S. cerevisiae. Out of thirty-three enzymes investigated, mainly by Halvorson and his fellow workers, thirty-two showed a periodic increase in activity (reviewed by Halvorson et al, 1971).

An early indication that the observed periodic increases in enzyme activity may have been an artifact induced by the synchronisation procedure was obtained by Bellino (1973) in an investigation of aspartate transcarbamoylase through the E. coli cell cycle. In this study synchronous cultures were prepared by the membrane elution or "baby machine" method (Helmstetter and Cummins, 1963). This synchronisation method has been reported to result in a reduced amount of metabolic disturbance compared with alternative procedures for synchronising E. coli cells such as selection of small cells on sucrose gradients (Bellino, 1973). In synchronous cultures of E. coli prepared

by membrane elution the increase in activity of aspartate transcarbamoylase was found to be continuous through the cell cycle. In previous studies which had used synchronous cultures prepared by either reinoculation of stationary phase cultures or selection of small cells on a sucrose gradient the increase in activity had been found to be periodic (Masters and Donachie, 1966; Kuempel et al, 1965). This result suggested that the previously observed periodicity for aspartate transcarbamoylase activity was an artifact caused by the synchronisation procedure rather than an intrinsic property of the cells.

Mitchison and his co-workers (Mitchison, 1977) carefully investigated the possibility that the previously reported step enzymes in the fission yeast S. pombe (Bostock et al, 1966) were an artifact caused by the method used to obtain synchronous cultures. Synchronous cultures of S. pombe were prepared by selection of small cells on sucrose gradients followed by reinoculation into fresh media. Particular attention was paid to the behaviour of asynchronous control cultures, which were prepared by remixing cells that had been separated on sucrose gradients and then inoculating into fresh medium at the same density as used for synchronous cultures. If the asynchronous controls gave "steps" in enzyme activity then the results obtained with synchronous cultures could not be interpreted. For the small number of enzymes that had shown a stepwise increase in activity in synchronous cultures it was found that in most cases the periodic increases also occurred in the asynchronous control cultures. In S. pombe, out of 20 enzymes investigated only two, thymidinemonophosphate kinase and nucleoside diphosphokinase; gave an unambiguous periodic stepwise accumulation of activity (Mitchison, 1977; Dickinson, 1983).

From experiments carried out using budding yeast recent evidence

suggest that at least for some of the "step enzymes" the observed periodicities were also artifacts caused by the method of synchronisation. For example, Creanor et al (1983) re-examined the increase in activity of β -glucosidase and β -galactosidase through the cell cycle in synchronous cultures of budding yeast. In earlier work it had been reported that both these enzymes were step enzymes (Sebastian et al, 1971). Creanor et al prepared synchronous cultures by size selection from an elutriating rotor, a method that has been shown to reduce metabolic perturbations (Creanor and Mitchison, 1979). In contrast to the earlier results, no stepwise increases in activity were found for either of the two enzymes.

A large amount of information exists on the patterns of macromolecular synthesis through the cell cycle of the unicellular algae Chlorella (reviewed by Tamiya, 1966). In synchronous cultures of Chlorella prepared by alternate light/dark cycles a number of different enzymes have shown periodic increases in enzyme activity during the cell cycle (reviewed by Lorenzen and Hess, 1974).

More recent evidence suggests that these periodic steps in enzyme activity result from perturbations caused by the synchronisation procedure. A synchronous culture of Chlorella obtained by periodic illumination was grown in a turbidostat under conditions of constant light and turbidity. During the second cell cycle under these conditions the periodic increases in enzyme activity were no longer observed. This suggested that the periodicities were not part of the cell cycle (John et al, 1981).

Two main conclusions can be drawn from the large amount of data gathered on the increase in enzyme activities through the cell cycle. Firstly, the activity of most enzymes seems to increase continuously through the cell cycle. Secondly, the methods used to obtain

synchronous cultures can induce artifactual periodicities in enzyme activity. In the absence of asynchronous controls these perturbations can be mistaken for cell cycle events. Therefore, it is important whenever possible, to test for perturbations by running suitable controls.

A different approach to identify the periodic synthesis of proteins involved a direct examination of polypeptides separated by electrophoresis on two dimensional gels. The relative rates of synthesis of individual proteins was determined by pulse labelling, usually with ^{35}S -methionine, synchronous or age fractionated asynchronous cultures. This approach has been used to study the synthesis of individual proteins through the cell cycle in a number of different organisms including *S. cerevisiae* (Elliot and McLaughlin, 1978), *E. coli* (Lutkenhaus et al, 1979), *Caulobacter crescentus* (Milhausen and Agabian, 1981), *P. polycephalum* (Turnock et al, 1981), *C. sarokiniana* (John et al, 1982) and HeLa cells (Milcarek and Zahn, 1978). From these studies no unequivocal periodically synthesised proteins were identified. In a recent careful study of polypeptide synthesis in budding yeast by Lorincz et al (1982) the rates of synthesis of 900 individual proteins were examined through the cell cycle. Of these, 20, including three histones, showed a periodic pattern of synthesis.

Although the great majority of proteins are synthesised continuously during the cell cycle a periodic increase in enzyme activity and or periodic protein synthesis have been shown to occur in a number of cell types when care has been taken to reduce synchronisation artifacts. In a number of different organisms such as mammalian cells or budding yeast the histone proteins have been shown to be synthesised periodically (Robbins and Borun, 1967; Moll and Wintersberger, 1976). The activity of some enzymes involved in DNA precursor metabolism have

also been found to increase periodically. For example, in S. pombe only two enzymes, thymidine monophosphate kinase and nucleoside diphosphokinase, out of 20 examined show a periodic stepwise increase in activity (Mitchison, 1977; Dickinson, 1983). In addition the activity of enzymes involved in DNA synthesis, such as DNA polymerase, also been shown to be cell cycle regulated in a number of organisms have (Cited by Mitchison, 1977). In P. polycephalum it was recently demonstrated that the tubulin proteins, which are important structural components of the mitotic spindle, are synthesised periodically during late G₂ (Laffler et al, 1981; Schedl et al, 1984).

In summary, a small number of proteins have been identified which show cell cycle periodicity. Most of these proteins are associated with periodic events in the cell cycle such as DNA synthesis or mitosis.

4 Cell Cycle Mutants

Cell cycle mutants have been isolated from a number of organisms ranging from prokaryotes through lower to higher eukaryotes (reviewed by Simchen; 1978). Most of these are temperature sensitive conditional lethal mutants. At the restrictive temperature cell division cycle (cdc) mutant cells are found, in general, to arrest at a unique stage in the cell cycle. After prolonged incubation at the restrictive temperature cdc mutant cells show a characteristic and sometimes abnormal phenotype. This is termed the terminal phenotype of the mutant and is used in characterising the mutation. In addition, a few non-lethal cell cycle mutants have been obtained which can complete the cell cycle but are altered in some way. For example, in S. pombe the wee mutants divide at a reduced cell size and are altered in the duration of the G_1 and G_2 phases compared to wild type cells (Nurse, 1975).

In the following section the methods used to determine the stage when a mutant gene product acts in the cell cycle and its dependency relations with other cell cycle events are discussed. Although these methods can be used in the characterisation of cell cycle mutants from any organism, they have been used most extensively in the genetic analysis of the cell cycle in budding and fission yeast. In the section that follows examples from S. cerevisiae and S. pombe will be considered exclusively.

(i) Temporal Order and Dependency

Cell cycle mutants have been used to generate a temporal map of cell cycle events. The most common method of determining when a mutant gene product is required is to calculate its transition point or execution point. The transition point is the time in the cell cycle beyond which a shift from the permissive to restrictive temperature can no longer prevent a mutant cell from successfully completing the ongoing cell cycle (Nurse et al, 1976). Although the timing of the transition point is open to ambiguous interpretation (discussed by Pringle, 1978) it can be used to give an estimate of when the wild type product functions in the normal cell cycle.

When the transition points of a number of cdc mutants were determined a definite pattern in their distribution through the cell cycle was observed. In most cases the time of the transition point was shortly before the cell cycle event with which the cdc mutation was associated. For example, the seven cdc genes in S. pombe that are associated with S phase all have transition points within 0.15 of a cycle before it occurs (Nasmyth and Nurse, 1981). Such a distribution could be explained if certain cell cycle events were rate limiting. When these rate limiting events were completed, other cell cycle functions, dependent on their completion, would take place rapidly accounting for the bunched distribution of transition points.

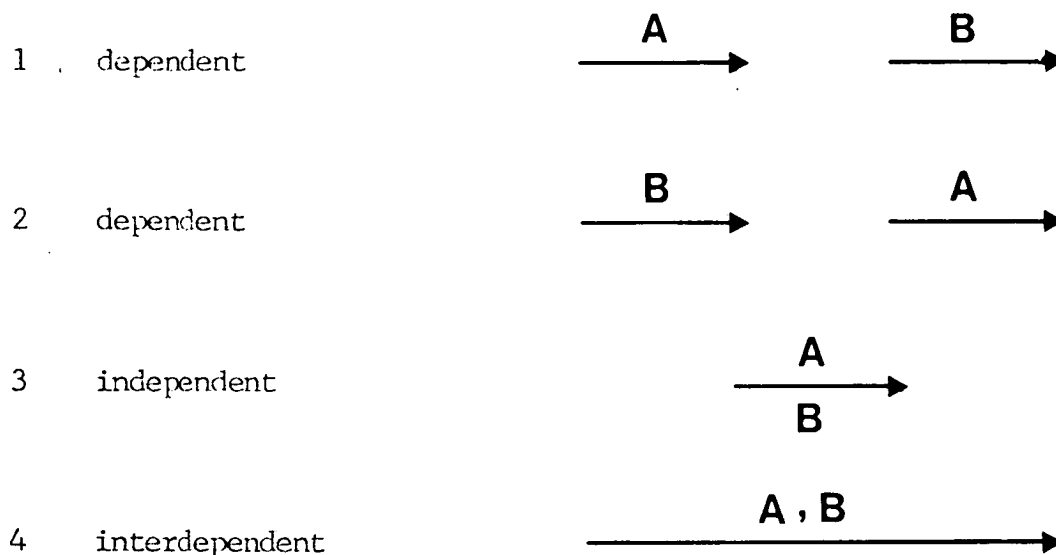
Such a temporal map gives no information about the dependency relationships between different cell cycle events. Such dependency relationships can sometimes be inferred from a careful examination of the terminal phenotypes of the various cdc mutants isolated. For example, S. pombe cdc mutants defective in early nuclear division were

also found not to initiate septation. In contrast cdc mutants defective in septum formation were able to complete successive rounds of DNA synthesis and nuclear division (Nurse et al, 1976). These observations led to a model of the S. pombe cell cycle incorporating two dependent sequences of events (see Figure 1.3; Section 1.6). A similar set of dependent sequences were inferred from S. cerevisiae cdc mutants (Hartwell et al, 1974).

Blocks defined by different cdc mutations can themselves be organised into dependent sequences. Two methods exist for determining the dependency relationship between gene products containing cdc mutations. The first involves the comparison of single with double mutant phenotypes. It is necessary that the terminal phenotypes of the two mutants can be distinguished and the restrictive conditions be applied simultaneously. If the two mutants are defective in sequentially dependent steps, the double mutant should have a terminal phenotype that is characteristic of the mutant whose gene product fails to function first. Such a method was used by Hereford and Hartwell (1974) to map the dependency relationships between cdc genes required for initiation of DNA synthesis in S. cerevisiae.

The second method termed the reciprocal shift requires two reversible blocks, A and B, that can be applied independently. The method requires two separate experiments. In the initial experiment block A is applied first. When all the cells are arrested, block A is removed and block B is administered. The second experiment is the reciprocal of the first. Block B is employed first followed by block A. The method differentiates four relationships between the two blocks which are shown in Figure 1.2. The reciprocal shift was used by Fantes (1982) to investigate the dependency relationships

Figure 1.2 Four possible relationships between two blocks differentiated by a reciprocal shift experiment



Four possible relationships between the two steps, A and B. The two steps could be dependent on one another, B dependent on A (1) or A dependent on B (2). Block A and B could be independent in which case either step can occur when the other is blocked. Alternatively the two steps may be interdependent where both steps occur together.

between different S. pombe cdc mutants that are defective in gene products required for mitosis.

An important conclusion from the results discussed above is that the observed temporal order of cell cycle events seems to be, at least in part, a consequence of functional dependency between the events rather than by an alternative mechanism involving, for example, a central timer.

(ii) Cell Cycle Controls

When proliferating **mammalian** cells are caused to arrest, for example by nutrient starvation, cells are exclusively found in the G_1 phase of the cell cycle (Pardee et al, 1978). Furthermore, when most mammalian cell types are grown at different growth rates the time taken to traverse S, G_2 and M phases are found to be relatively constant (Prescott, 1976). The differences in generation times can be almost totally explained by an increase in the time taken to complete the G_1 phase. Such information suggests that one or more major cell cycle control points exist in G_1 for mammalian cells.

Similarly, in budding yeast, evidence exists for a major cell cycle control point in G_1 (Reviewed in Hartwell, 1973). This control point is called "start" and is thought to be the point at which the commitment to the mitotic cell cycle occurs rather than other alternative developmental pathways (discussed in the next section). A number of studies have indicated that a size control is associated with "start" (Johnston et al, 1977; Johnston et al, 1979).

Not all cell types studied have been found to have their primary cell cycle control point in G_1 . In several cell types that contain

little or no G_1 phase the major control seems to occur in G_2 . For example, studies on the Amoeba cell cycle have shown that a size control appears to operate in G_2 (Ron and Prescott, 1969). A G_2 size control has also been reported for Physarum (Loidl and Sachsenmaier, 1982).

In the fission yeast S. pombe the major cell cycle control point also occurs in G_2 . This has been shown to be a size control required for the initiation of mitosis (Nurse, 1975; Thuriaux, et al, 1978). Some recent experiments suggest that an analogous control to "start" in budding yeast operates in the G_1 phase of fission yeast (Nurse and Bisset, 1981 ; discussed in next section). Cell size may also play a role in the S. pombe "start" since it has been demonstrated that a critical size must be attained before DNA replication can be initiated (Fantes and Nurse, 1978). The G_1 size control is cryptic in wild type cells as they are usually above this critical cell size at the beginning of the cell cycle.

Some of the best evidence for the existence of the above control points being involved in the regulation of the S. pombe cell cycle has come from investigations carried out using cell cycle mutants. These are discussed in the next section.

(iii) Commitment to the Mitotic Cell Cycle

From studies originally carried out in S. cerevisiae it has been shown that the products of certain cdc genes, termed "start" genes, are required at some early event in G_1 shortly before the cell becomes committed to the mitotic cell cycle. This event has been called "start" and before a cell can pass this point it monitors various parameters

such as nutritional conditions, attainment to a critical cell size, the presence or absence of factors required for conjugation and sporulation and the completion of the previous mitosis. On the basis of this information, the cell becomes committed to a particular developmental pathway once it passes "start".

Cell cycle mutants have provided important evidence for the presence of such a commitment point. In S. pombe, the cdc 2 and cdc 10 genes are required during G_1 before the initiation of DNA replication. If cells with mutations in the above genes are arrested at the restrictive temperature, they are still able to conjugate with cells of the opposite mating type (Nurse and Bisset, 1981). Cells which arrest at later cdc gene steps are unable to do so. This suggests that cells choose between entering the mitotic cycle or conjugation only at "start" and not at other points in the cell cycle.

(iv) Initiation of Mitosis

This work has mainly concentrated on the characterisation of S. pombe cell size or wee mutants whose most obvious phenotype is a reduction of cell size at division (Nurse, 1975). These studies have shown that wee mutants are defective in a control which determines a cell size requirement for entry into mitosis. The wee mutants isolated map in two genes wee 1 and cdc 2. Previous work had identified mutations in the cdc 2 gene that conferred a conditional lethal cdc phenotype (Nurse et al, 1976).

Genetic analysis of wee 1 mutants has suggested that the wee 1 gene product acts as an inhibitor of mitosis. Therefore, when the wee 1⁺ product is inactivated, cells are advanced into mitosis at a reduced cell

size. (Nurse and Thuriaux, 1980).

From similar work on cdc 2 mutants it has been proposed that the cdc 2 gene product functions as an activator for the initiation of mitosis. Destroying the activator, as in conditional lethal cdc mutations, prevents mitosis occurring, while some form of modification as in wee mutants of cdc 2 advances cells into mitosis at a reduced cell size (Nurse and Thuriaux, 1980).

5 Identification of cdc gene products

Genetic analysis has been successful in identifying a large number of genes required for cell cycle progress. The long term aim of such studies is to gain an understanding of the molecular interactions involved in different cell cycle events. To achieve this objective depends on the identification of protein products coded by cdc genes. Unfortunately the biochemical function defective in most cdc mutants is unknown. In S. pombe out of greater than twenty five genes identified the protein products of only four are known. The cdc 17 gene codes for DNA ligase (Nasmyth, 1977). The cdc 22 gene has been reported to code for nucleoside diphosphokinase (Dickinson, 1981), while nda 2 and nda 3 code for α and β -tubulin. (Toda et al, 1984; Hiraoka et al, 1984).

The recent development of recombinant DNA techniques and efficient methods of DNA transformation, first for S. cerevisiae and more recently for S. pombe, has offered a promising new approach towards the identification of cdc gene products. (Hinnen et al, 1973; Beach and Nurse, 1981). Chimeric plasmid vectors are available **that are capable of replication** in yeast and E. coli (Beggs, 1978; Beach et al, 1982). Into these vectors random wild type yeast DNA fragments have been inserted

constituting a "gene library" of the yeast genome. A number of yeast cdc genes have been cloned from such libraries by selecting hybrid plasmids containing a DNA fragment including the cdc⁺ gene. These plasmids were selected on the basis of their ability to complement particular cdc mutations in transformed yeast cells (Masmyth and Reed, 1980; Beach et al, 1982). The cloning of a gene allows a number of approaches to be used in the identification of the gene product.

The nucleotide sequence of the cloned gene can be determined. This provides information on gene structure such as the presence or absence of **introns** as well as an estimate of the size of the polypeptide coded by the gene. The sequence can also be **scanned** by computer for the presence of known "consensus sequences". A consensus sequence is a general nucleotide sequence that has been recognised empirically to be involved in specific biological functions such as ATP or DNA binding sites. The presence of such a sequence could suggest the possible biological function of the gene product in the cell. The nucleotide sequence of the cloned S. pombe cdc 2 gene contains the consensus sequences for the ATP binding and phosphorylation acceptor sites of protein kinases such as bovine **CAMP**-dependent protein kinase and the src family of oncogene products (Hindley and Phear, 1984). However although the presence of a consensus sequence can suggest the possible biological function of a cloned gene this still has to be confirmed by investigating the biochemical properties of the protein product coded by it.

The total nucleotide sequence can also be compared with those stored in a computer database bank to detect any homology with genes that have previously been sequenced. Such **comparisons** are particularly useful when a conserved protein is involved. In S. pombe, the gene

product of the nda 2 gene was recently established to be α -tubulin by such a method (Toda et al, 1984).

Another use of cloned cdc genes is as a hybridisation probe to detect the transcripts coded by the gene. If RNA obtained from synchronous cultures is probed using the cloned cdc gene the cell cycle regulation of transcript levels can be investigated.

The availability of a cloned gene allows a number of different approaches to be taken in the characterisation of the gene product. The corresponding mRNA can be isolated by hybridisation and translated in vitro (Pelham and Jackson, 1976). The translation products can be separated by electrophoresis to determine the size of polypeptide coded by the cloned gene. Alternatively, the DNA containing the cloned DNA fragment can be inserted in front of an efficient promoter in an expression vector. Expression vectors based on lambda virus (Murray et al, 1979) or plasmid vectors (Brosius, 1984) are available for overproduction of proteins in E. coli. Plasmid vectors have also been described for overexpression of proteins in both S. cerevisiae and S. pombe (Johnston and Davis, 1984; Russell and Woodbury, 1983). Using such expression vectors a large amount of the cdc protein product can be obtained making subsequent purification easier. Biochemical studies can then be carried out on the purified protein. The purified gene product can also be used to raise specific antibodies against it. An antibody is a useful reagent that can be used to detect the cdc product present in crude cell extracts. For more quantitative measurements, radioimmuno assays can be used. In addition, the antibody can be used in cytological studies to determine the intracellular location of the gene product by immunofluorescence (Hiraoka et al, 1984).

The work described in this thesis involved an investigation of the cdc 22 gene of S. pombe. In the remainder of the introduction there follows a summary of the main features of the S. pombe cell cycle followed by a review of previous studies on the cdc 22 gene.

6 The S. pombe Cell Cycle

S. pombe is a rod shaped organism that grows in length without much change in diameter. Cell division divides the mother cell in half into two equal daughters. These **properties** make S. pombe an ideal choice to study the co-ordination between growth and cell division as the length of the organism gives a good estimate of cell size (Mitchison, 1957).

The cell cycle of S. pombe is similar to higher eukaryotes containing a short G_1 that is about 0.1 of a cycle, a short S phase also about 0.1 of a cycle, followed by a long G_2 that lasts about 0.75 of a cycle (Nasmyth et al, 1979). During mitosis in S. pombe, as in most fungi, the nuclear membrane does not break down and the chromosomes do not normally visibly condense (McCully and Robinow, 1971). This makes an estimate of the time taken to complete mitosis difficult though it is thought to be short, lasting about 0.05 of a cycle (Cited in Fantes, 1984). Cell division is delayed compared to higher eukaryotes occurring about 0.25 of a cycle after mitosis. Thus cells have completed G_1 before cell division has taken place (Nasmyth et al, 1979).

A number of cell cycle mutants have been isolated in this organism. Characterisation of these mutants using the methods discussed previously (see Section 1.4 (iv)), has led to a model of the S. pombe cell cycle combining two dependent sequences of events (Figure 1.3).

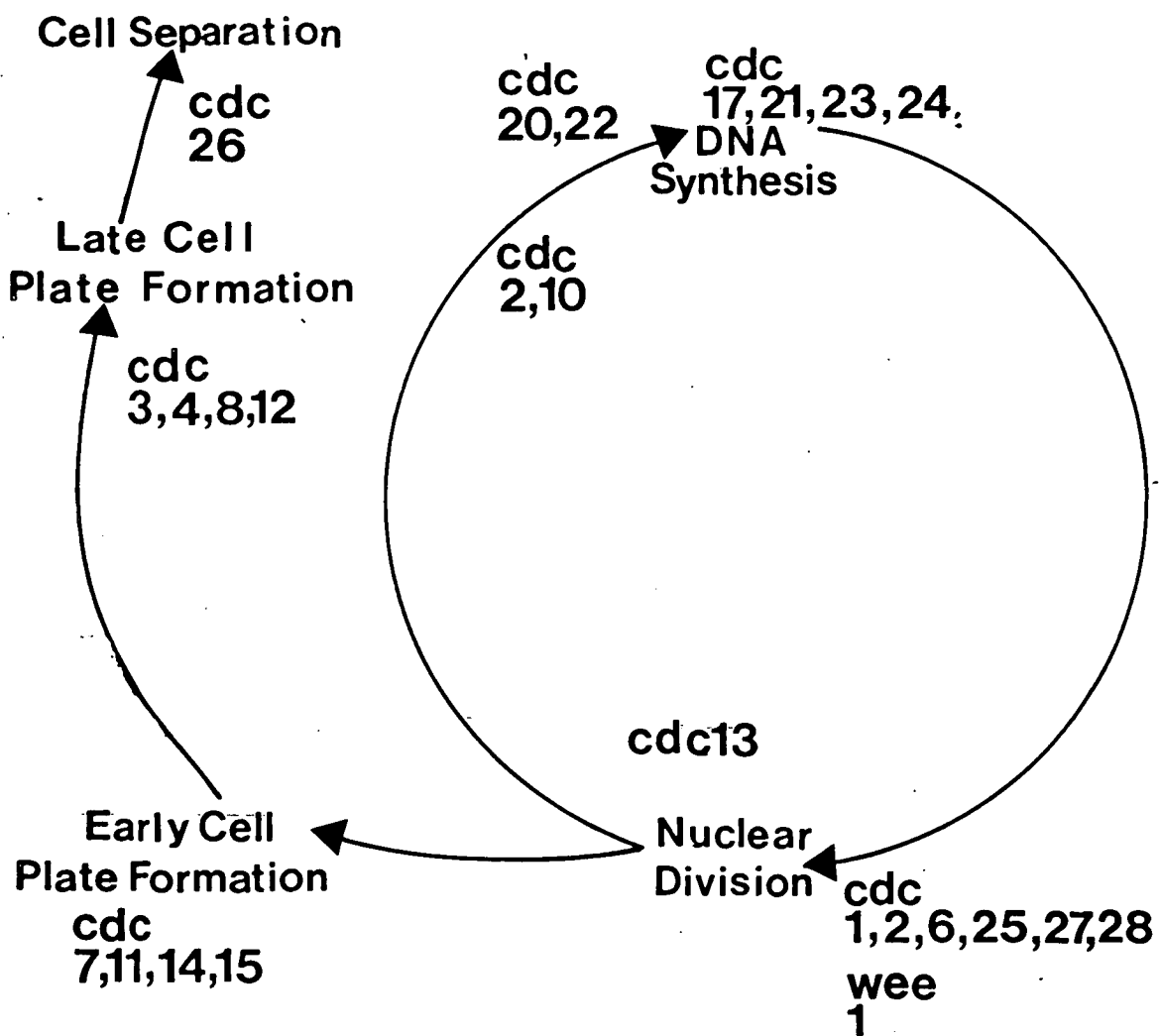
7 S. pombe Cell Cycle Mutants Defective in S Phase

Nasmyth and Nurse (1981) isolated cdc mutations that were defective in the initiation or elongation of DNA replication. These mutants were characterised by following the pattern of DNA synthesis after shift from the permissive to the restrictive temperature in synchronous culture. The dependency of the cdc mutant function relative to a hydroxyurea block was also examined. As hydroxyurea inhibits DNA replication (Mitchison and Creanor, 1971) the dependency relationship of the cdc mutation relative to S phase was elucidated.

The cdc mutant was first arrested with hydroxyurea at the permissive temperature and then incubated in the absence of the drug at the restrictive temperature. The reciprocal experiment of imposing the cdc block followed by a hydroxyurea block was not carried out. The experiment determined if the cdc mutant function occurred either :-
 (i) before or independently of the hydroxyurea sensitive step, or
 (ii) after or with the hydroxyurea block.

From these experiments it was concluded that two genes cdc 20 and cdc 22 were required for the initiation of DNA synthesis. A previous study had demonstrated that an additional gene, cdc 10, was also required for the initiation of S phase (Nurse et al, 1976). Two genes, cdc 21 and cdc 23, were thought to be involved in DNA chain elongation, while cdc 24 and cdc 17 were concerned with the joining

Figure 1.3 Dependent sequences of cell cycle gene-controlled steps in *S. pombe*



of replicons. Further investigation of a cdc 17 mutant strain showed that it had a temperature sensitive defect in the structural gene for DNA ligase (Nasmyth, 1977).

8 Biochemical Characterisation of the cdc 22 Mutation

The biochemical characterisation of S. pombe cdc mutants required for DNA replication indicated that a mutation in the cdc 22 gene caused a temperature defect in the enzyme nucleoside diphosphokinase (NDPK) (Dickenson, 1981). This enzyme is required for nucleotide biosynthesis and catalyses the conversion of NDP to NTP, where N is any ribo or deoxyribonucleotide (Parks and Agarwal, 1973). The evidence for cdc 22 encoding NDPK activity is described in some detail below, due to its importance to the project as a whole.

Preliminary evidence for cdc 22 mutant cells being defective in NDPK came from an examination of nucleotide pools in wild type and cdc 22 - M45 strains after shifting from the permissive to the restrictive temperature. Wild type and cdc 22 - M45 cells were shifted to the restrictive temperature of 36°C and pulse labelled with [5, 6 ³H] uridine. The pattern of radioactivity obtained in the nucleotide pools isolated from wild type and mutant cells were then compared. In the wild type strain the temperature shift caused a small increase in the radioactivity found in dTTP with a corresponding fall in the radioactivity obtained in dTDP. In the cdc 22 - M45 strain incubation at the restrictive temperature caused a marked reduction in the amount of radioactivity found in dTTP, while the quantity of radioactivity in dTDP increased dramatically. This suggested that the cdc 22 - M45 strain was defective in the enzyme that catalysed the conversion of

dTDP to dTTP, nucleoside diphosphokinase (NDPK).

To test this hypothesis the activity of NDPK was measured in crude cell extracts obtained from wild type and cdc 22 - M45 cells. It was found that the NDPK activity obtained from cdc 22 - M45 cells was 1.6 times less than from wild type cells when measured at the physiological restrictive temperature of 36.8°C. However, a much more dramatic demonstration of the temperature lability of NDPK from cdc 22 - M45 cells was observed when the K_m values were calculated. At the restrictive temperature the K_m value for TDP of NDPK obtained from cdc 22 - M45 cells was 11 times greater than that obtained from wild type cells.

Additional work by Dickinson (1983) focussed on the increase in activity of NDPK through the cell cycle. When NDPK activity was measured in synchronous cultures of wild type cells it was found to be periodic showing a stepwise doubling of activity with a mid-point at 0.61 of a cycle. In synchronous cultures of wee 1.6 mutant cells NDPK was also observed to increase in a periodic stepwise manner. The mid-point of the rise occurred 0.35 of a cycle later than in wild type cells. This was thought to be a reflection of S phase being delayed in wee 1.6 cells occurring about 0.3 of a cycle later than in wild type cells (Nasmyth et al, 1979).

In addition, the mutant strains cdc 2.33 and cdc 10.129 were used to investigate whether a continued rise in NDPK activity was dependent on cell cycle progress. In asynchronous cultures of both mutant strains a shift up to the restrictive temperature results in a rapid inhibition of DNA replication. However, when both these mutant strains were shifted

to the restrictive temperature the activity of NDPK continued to increase exponentially. This suggested that cell cycle progress was not required for an increase in NDPK activity.

The original aim of this thesis was to isolate the cdc 22 gene of S. pombe. As discussed in the last section it was thought that the cdc 22 gene coded for the enzyme nucleoside diphosphokinase (NDPK) which showed a periodic step in activity during the cell cycle. (However recent evidence has brought this gene/enzyme relationship into doubt; see Chapter 9). The cloned cdc 22 gene was to be used as a specific hybridisation probe to investigate if the observed periodicity in NDPK activity was regulated by the amount of the cdc 22 transcript present.

MATERIALS AND METHODS

1 Schizosaccharomyces pombe

(i) Strains used in this work

The strains of S. pombe used in this work were derived from the wild type stock of Leupold (Leupold, 1950), 972 heterothallic h^- and 975 heterothallic h^+ . The wild type strains are both prototrophic.

The mutant alleles used are shown in Table 2.1 along with a description of their phenotype.

(ii) General methods for handling S. pombe

The methods used follow those described by Gutz et al, 1974 and Kohli et al, 1977. Strains were grown at 25°C unless otherwise stated.

Strains of S. pombe were stored for up to a few months on yeast extract slopes at 4°C. Long term storage was carried out on silica gels at 4°C as described by Gutz et al, 1974.

(iii) Media and growth conditions

(a) Malt extract media (ME)

This has been previously described by Gutz et al, 1974. It was used for crosses between strains of opposite mating types. The components are shown in Table 2.2.

Table 2.1

GENE	ALLELE	PHENOTYPE	REFERENCE
<u>ade</u> 6	7G4	adenine auxotroph	Kohli <u>et al</u> , 1977
<u>cdc</u> 1	7	defective in mitosis at 35°C	Nurse <u>et al</u> , 1976
<u>cdc</u> 2	33	defective in mitosis at 35°C	Nurse <u>et al</u> , 1976
<u>cdc</u> 10	129	defective in the initiation of DNA replication at 35°C	Nurse <u>et al</u> , 1976
<u>cdc</u> 17	M75	defective in DNA replication at 35°C	Nasmyth <u>et al</u> , 1981
<u>cdc</u> 20	M10	defective in the initiation of DNA replication at 35°C	Nasmyth <u>et al</u> , 1981
<u>cdc</u> 21	M68	defective in DNA replication at 35°C	Nasmyth <u>et al</u> , 1981
<u>cdc</u> 22	M45	defective in the initiation of DNA replication at 35°C	Nasmyth <u>et al</u> , 1981
	C11	defective in the initiation of DNA replication at 35°C	Creanor, J. Pers Comm
<u>cdc</u> 23	M36	defective in DNA replication at 35°C	Nasmyth <u>et al</u> , 1981
<u>cdc</u> 24	M38	defective in DNA replication at 35°C	Nasmyth <u>et al</u> , 1981
<u>leu</u> 1	32	leucine auxotroph	Kohli <u>et al</u> , 1977
<u>mat</u> 2	102		Egel, 1973
<u>cdc</u> - C1	-	defective in DNA replication at 35°C	Creanor, J. Pers Comm
<u>cdc</u> - C2	-	defective in DNA replication at 35°C	Creanor, J. Pers Comm
<u>cdc</u> - C4	-	defective in DNA replication at 35°C	Creanor, J. Pers Comm
<u>cdc</u> - C41	-	defective in DNA replication at 35°C	Creanor, J. Pers Comm
<u>cdc</u> - C43	-	defective in DNA replication at 35°C	Creanor, J. Pers Comm
<u>cdc</u> - C47	-	defective in DNA replication at 35°C	Creanor, J. Pers Comm
<u>wee</u> 1	6	small cells	Nurse, 1975

Table 2.2 Malt Extract Agar

	<u>g/l</u>
Malt Extract (Difco)	30
Agar (Difco)	20

Table 2.3 Yeast Extract Agar

	<u>g/l</u>
Yeast Extract (Difco)	5
Glucose (BDH)	30
Agar	20

Table 2.4 Edinburgh Minimal Media 2

	<u>g/l</u>
Potassium Hydrogen Phthalate	3
Di Sodium Hydrogen Orthophosphate x 2 H ₂ O	1.8
Ammonium Chloride	5
Glucose	20
Salts Stock	20 mls
Vitamins Stock	1 ml
Trace minerals stock	0.1 ml
Potassium Hydroxide (1M)	1 ml (for solid media only)

Salt Stock Solution

	<u>g/l</u>
Magnesium Chloride	53.5
Calcium Chloride	1
Potassium Chloride	50
Sodium Sulphate	2

Vitamins Stock Solution

	<u>g/l</u>
Inositol	10
Nicotinic acid	10
Calcium pantothenate	1
Biotin	10 mg
	<u>g/200 mls</u>

Trace Minerals Stock

H_3BO_3	1
$MnSO_4 \times 4H_2O$	1
$ZnSO_4 \times 7H_2O$	0.8
$FeCl_3 \times 6H_2O$	0.4
H_2MnO_4	0.288
$CuSO_4 \times 5H_2O$	80 mgs
Citric acid	2 mgs
KI	20 mgs

(b) Yeast extract (YE)

This was the complex medium described by Gutz et al, 1974 used for the routine growth and isolation of strains. Its composition is given in Table 2.3.

(c) Edinburgh minimal media 2 (EMM)

EMM 2 as modified by Nurse (1975), was the defined minimal media used in all cell physiology experiments. The salts, vitamins and trace minerals were all stored separately as stock solutions and added in amounts shown in Table 2.4 prior to autoclaving.

In growth of cells for transformation experiments its composition was modified by adding 0.5% glucose instead of 2% as described in Beach et al, 1982. For regeneration of protoplasts during transformation experiments the media was made isotonic to the protoplasts by the addition of sorbitol to a concentration of 1.2 M. The composition of the above media are given in Table 2.5.

(d) Solid media

2% Bacto-Difco agar was added to the liquid media before autoclaving. Sometimes Phloxin B (Sigma) which stains dead cells, was added from a sterile stock solution at 20 mg/l, after the autoclaved media had cooled below 60°C.

Table 2.5 Osmotically stabilised EM

As EM 2 but included per litre.

Potassium Chloride	15 g
Sorbitol	109 g

Table 2.6 YEPD

	<u>g/l</u>
Glucose	30
Yeast extract	5
Peptone (Difco)	5

(e) Yeast extract peptone dextrose (YEPD)

This was the standard complex liquid medium used for growing yeast strains for DNA isolation. It was used as described by Yamamoto, 1980. Its components are shown in Table 2.6.

(iv) Cell Physiology

(a) Temperature shift experiments

Strains for temperature shift experiments were first checked to determine if they had the correct phenotype. A single colony was used to inoculate a 10 ml EMM preculture. The preculture was incubated at the permissive temperature until stationary phase had been reached. An aliquot of the stationary phase culture was used to inoculate 200 mls of EMM in a 500 ml conical flask. The culture was incubated at the permissive temperature until an optical density of 0.1 to 0.2 (at λ 595) was reached.

The culture was then divided into two equal parts; one half remained at the permissive temperature while the other was shifted to the restrictive temperature. Samples were removed from both cultures at specific times after the shift for determination of cell number and optical density. Transition points were estimated as described by Nurse et al, 1976.

(b) Cell number

Cell number was determined from a 500 μ l sample taken with a constriction pipette. Samples were fixed in a filtered 0.1% formaldehyde/

0.1% NaCl solution and cells counted with a Coulter (model B) particle counter as described by Mitchison, 1970.

(c) Optical Density

Optical density was measured in a Pye Unicam SP600 spectrophotometer from the absorbance at λ 595 nm of a 3 ml sample in a quartz cuvette.

(d) Synchronous Cultures

Synchronous cultures of S. pombe 972 wild type cells were prepared by selecting small cells from a Beckman JE-6 elutriator rotor.

An aliquot of stationary phase cells from a 10 ml EMM preculture was used to inoculate one litre of EMM. The culture was incubated at 35°C overnight in a constant temperature room. Vigorous agitation was provided by a LK fermentation MK orbital shaker to ensure that clumping of cells did not occur. The culture was used to prepare a synchronous culture when an optical density of 0.4 to 0.6 at 595 nm was reached. The protocol used was similar to that described by Creanor and Mitchison (1979). Cells were pumped at a flow rate of 25 ml min⁻¹ into the rotor which was spinning at a constant 4000 rpm in a Beckman J-21 centrifuge. Loading the yeast cells into the rotor routinely took between 15 to 30 minutes depending on the original density of the culture. To obtain a synchronous culture the small cells at the top of the elutriator cell were harvested by increasing the pump speed by 10-15% while leaving the rotor speed unchanged. A sample was checked under the microscope to estimate the degree of synchrony. If no cell plates could be observed in the yeast cells the synchrony of the

culture was judged adequate to use in an experiment. Cells were removed from the synchronous culture twenty minutes for cell number determinations and RNA extraction. Cell number was estimated from a 0.1 ml sample on a Coulter counter (See Chapter 2, Section 1(iv)b). RNA was extracted from samples containing approximately 2×10^7 cells. The cells were harvested on Oxoid 0.45 μm^3 filters, washed twice with cold 150 mM NaCl and immediately frozen at -20°C . To extract RNA the cells were thawed, resuspended in 0.5 ml of RNA extraction buffer (2% TNS; 50 mM Tris-HCl pH 7.5; 10 mM EDTA; 1 mg/ml DTT) and RNA prepared by the method described in Chapter 2 Section 4 (ii) d.

(v) Genetics

Standard genetical procedures were used for S. pombe by Gutz et al, 1974 and Kohli et al, 1977. To cross strains fresh isolates of opposite heterothallic mating type, + and -, were mixed together on the surface of a malt extract agar plate and left for three days at 25°C to allow for the formation of asci.

These asci could be used for either tetrad analysis or random spore analysis.

(a) Tetrad analysis

A loopful of asci was streaked out on the surface of a YE plate and single asci pulled using a glass needle attached to a Leitz micro-manipulator. The plate was then incubated at 20°C overnight to allow the asci to burst releasing its spores. These spores were then pulled apart from one another and incubated at 25°C until colonies formed.

(b) Random spore analysis

A loopful of asci was suspended in a 1:500 dilution of "Suc d' Helix pomatia" (an extract of snail gut enzyme obtained from Industrie Biologique Francaise S.A.) in sterile distilled water and incubated at 35°C overnight. The spores were collected by centrifugation and resuspended in sterile distilled water. The spore concentration was estimated by counting on a haemocytometer (Gelman Hawksley Ltd) and an estimated 200 spores were plated onto YE agar. The plate was then incubated at 25°C until colonies formed.

(c) Complementation tests

Complementation tests for allelism of pairs of cdc mutations were performed by crossing cdc x leu 1.32 ade 6.704 h⁻ to mat 2.102 lys 1.31 cdc y. The mating type allele mat 2.102 produces non-sporulating diploids when crossed to an h⁻ strain. (Egel, 1973). Diploids were selected on EMM at the permissive temperature and tested for complementation of the cdc phenotype at the restrictive temperature, 35°C.

(d) Haploidisation

The cdc gene to be assigned to a linkage group was crossed into a leu 1.32 ade 6.704 h⁻ strain and crossed to mat 2.102 lys 1.131 to give a diploid heterozygous (cdc⁺/cdc⁻) for the cdc mutation.

Haploidisation was carried out by transferring a diploid colony to YE + 10 µg/ml benomyl (Dr D Roy, Microbiology, Edinburgh University)

and incubated at 25°C overnight. A loopful of cells was suspended in sterile water and serially diluted for single colonies on YE + Phloxin B at 25°C. Pink haploid colonies were tested for cosegregation of one of the chromosomal markers lys 1 linkage group I; leu 1 linkage group II and ade 6 linkage group III with the temperature sensitive cdc phenotype.

2 Escherichia coli

(i) Strains used in this work

The recA E. coli strain JA221 (obtained from P. Nurse) was used routinely for molecular genetics experiments. The phenotype of this strain was recA1, leuB6, trpE5, hsdR⁻, hsdM⁺, lacY600.

However the recBC E. coli strain BJ.5183 (obtained from P. Nurse) was used for recovery of plasmids from S. pombe transformants. The phenotype was r⁻, recBC⁻, sbcB, endo, IGal⁻, meth⁻, strR, thi, biot, hsd.

(ii) General methods for handling E. coli

E. coli was handled as described in Maniatis et al, 1982. Strains were kept for up to a month on L. Broth agar plates at 4°C. Long term storage was in medium containing 15% glycerol at -70°C. Cells were grown at 37°C unless otherwise stated. Cell growth was estimated by optical density on a Unicam SP600 spectrophotometer.

(iii) Media

(a) L-Broth

This was the complex media used to reisolate and grow E. coli. Components are listed in Table 2.7.

(b) M-9 medium

This was the defined media used when testing for leucine auxotrophs. JA221 the strain routinely used was also auxotrophic for tryptophan which was added as a supplement at 2 mg/ml.

The media was made up from four stock solutions whose composition is shown in Table 2.8. Those stock solutions were added in the amounts shown in Table 2.8 to obtain M9 minimal media.

(c) Solid media

1.5%Bacto-Difco agar was added before autoclaving.

(d) Antibiotics

Antibiotic resistance was used as a selectable marker for bacteria carrying a plasmid. Antibiotics were added after the autoclaved media had cooled to below 60°C. Table 2.9 shows the final concentrations used to test for resistance.

Table 2.7 L-Broth

	<u>g/litre</u>
Bacto tryptone (Difco)	10
Yeast extract (Difco)	5
NaCl	10

Table 2.8 - M9 MEDIUMStock solution A

	<u>for 100 mls</u>
$\text{CaCl}_2 \times 6\text{H}_2\text{O}$	0.219 g
$\text{MgSO}_4 \times 7\text{H}_2\text{O}$	2.46 g

Stock solution B

	<u>for 1 ltr</u>
Na_2HPO_4	60 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g

Stock solution C

Thiamine chloride x HCl 0.1 g/100 mls

Stock solution D

Glucose 40 g/100 mls

Stocks A, B, D sterilised by autoclaving.

Stock C sterilised by filtering.

To make up 1 ltr of M9 media 10 mls A, 100 mls B; 1 ml C; and 5 mls D made up to 1 ltr with sterile distilled water.

Table 2.9 - Antibiotic Concentrations

	<u>Concentration (mg/ml)</u>
Ampicillin (Sigma)	50
Chloramphenicol (Sigma)	10
Tetracycline (Sigma)	12.5

3 Transformation

(i) Vectors

(a) pDB248

The vector pDB248 was derived from the E. coli plasmid pBR322 (Bolivar et al, 1977) and the S. cerevisiae chimeric plasmid pJDB248 (Beggs, 1979). It is capable of autonomous replication in E. coli and S. pombe. The Leu 2⁺ gene of S. cerevisiae complements the leu 1 gene of S. pombe and the leu B6 gene of E. coli. Therefore this marker can be used for selection in S. pombe and E. coli. The plasmid also contains antibiotic resistance genes for ampicillin and tetracycline for selection in E. coli. A restriction map of the plasmid is shown in Figure 2.1. (Beach and Nurse, 1982).

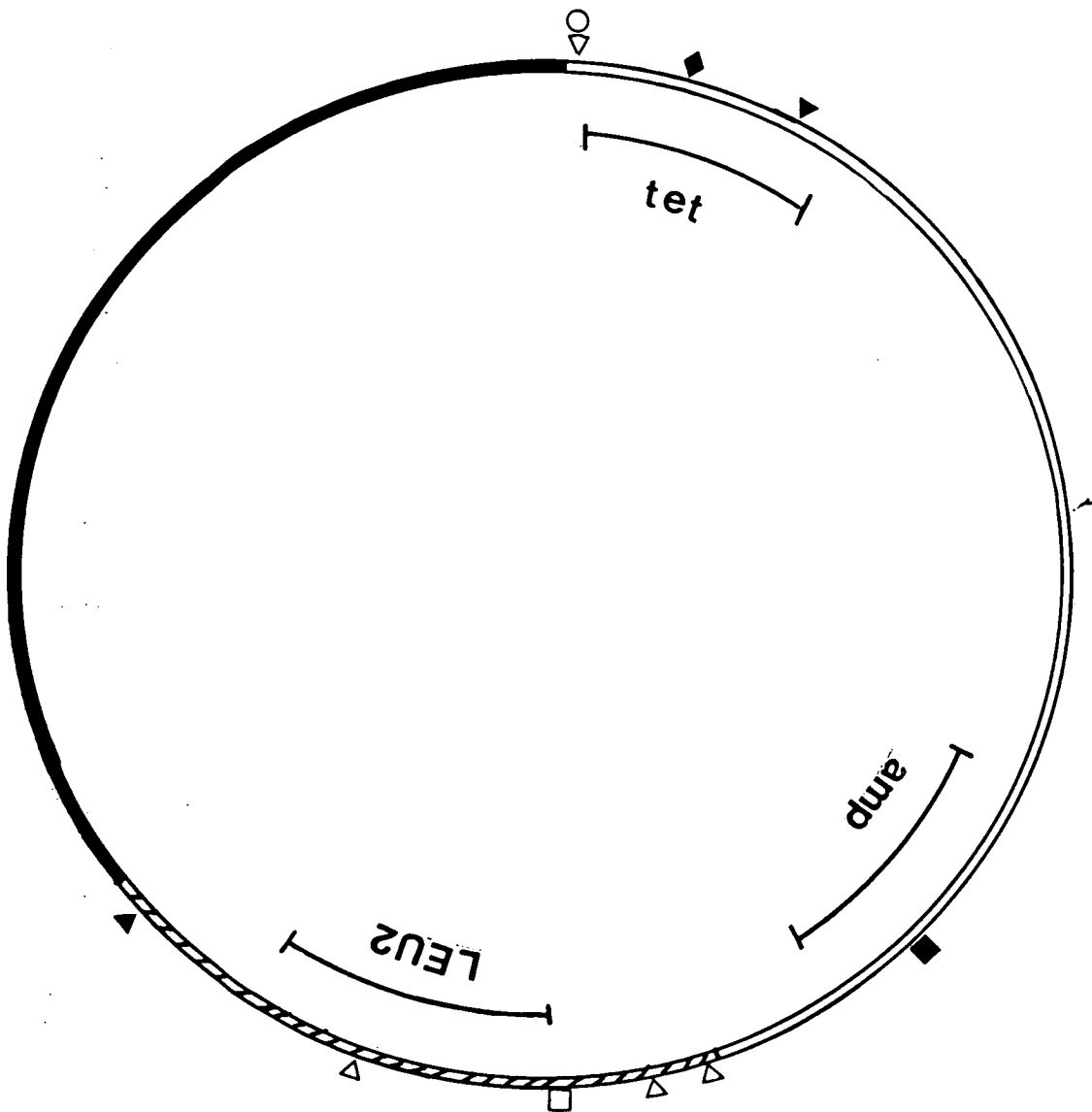
(b) pDB262

The vector pDB262 was constructed from the bacterial plasmid pTR262 (Roberts et al, 1980) and the S. cerevisiae sequences of pDB248. It is capable of autonomous replication in E. coli and S. pombe. This plasmid allows positive selection of DNA fragments inserted into the single HindIII or BclI restriction enzyme sites as only when fragments are inserted does the plasmid confer tetracycline resistance on E. coli. The vector also carries the Leu 2⁺ gene of S. cerevisiae for selection in S. pombe. A restriction map is shown in Figure 2.2.




(c) pDAM6 and pESPI

The plasmid pDAM6 was constructed by inserting the PstI fragment

Figure 2.1 Restriction map of pDB248.



Size: 10.6 Kb

-  Bacterial DNA sequences.
-  *S.cerevisiae* genomic DNA sequences.
-  2µm plasmid DNA sequences.
- amp** Ampicillin resistance.
- tet** Tetracycline resistance.
- LEU2** *S.cerevisiae* *LEU2* Gene.







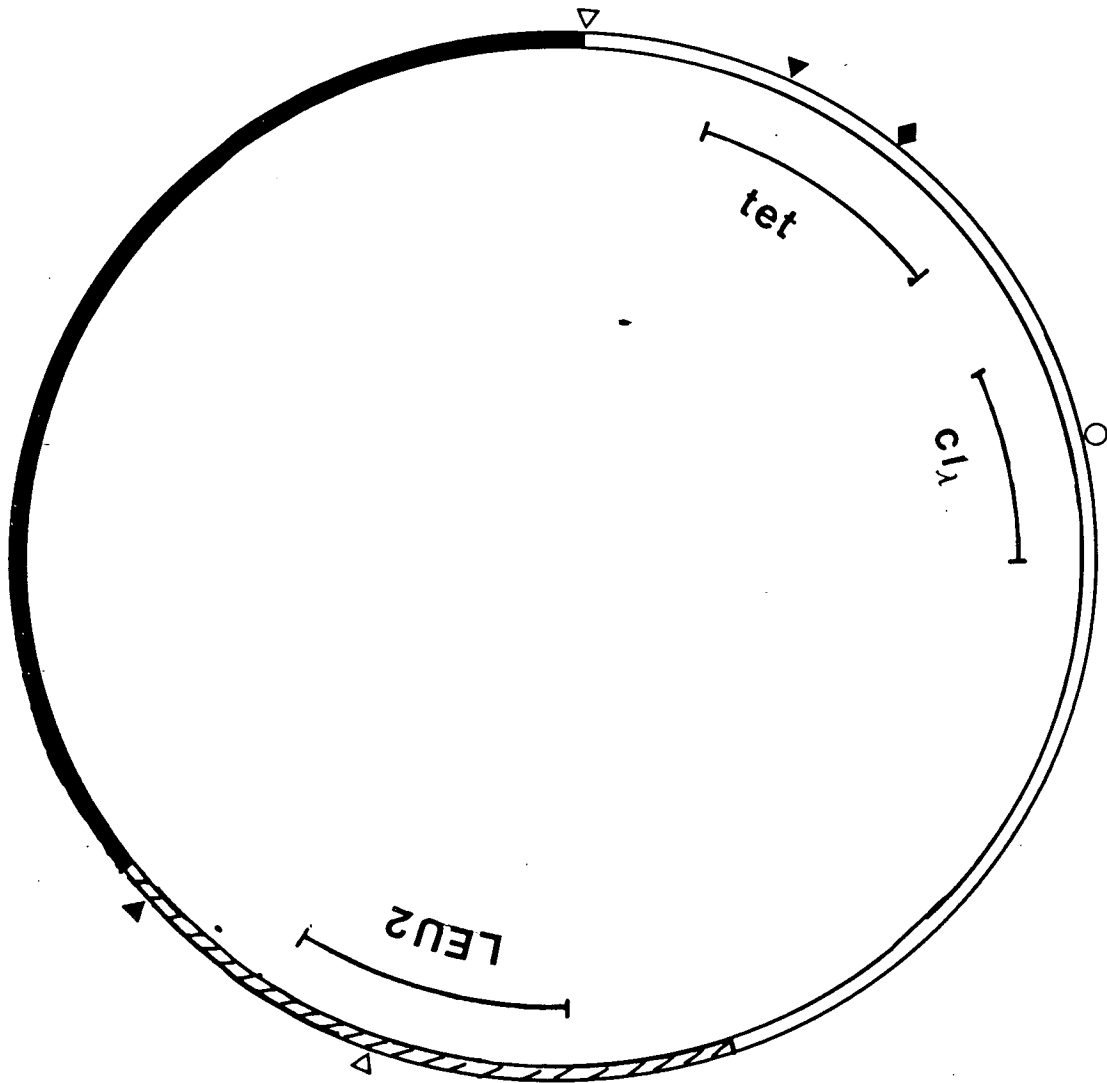
-  BamHI
-  EcoRI
-  HindIII
-  FstI
-  SalI
-  XhoI

Figure 2.2 Restriction map of pDB262.



Size: 10.6Kb








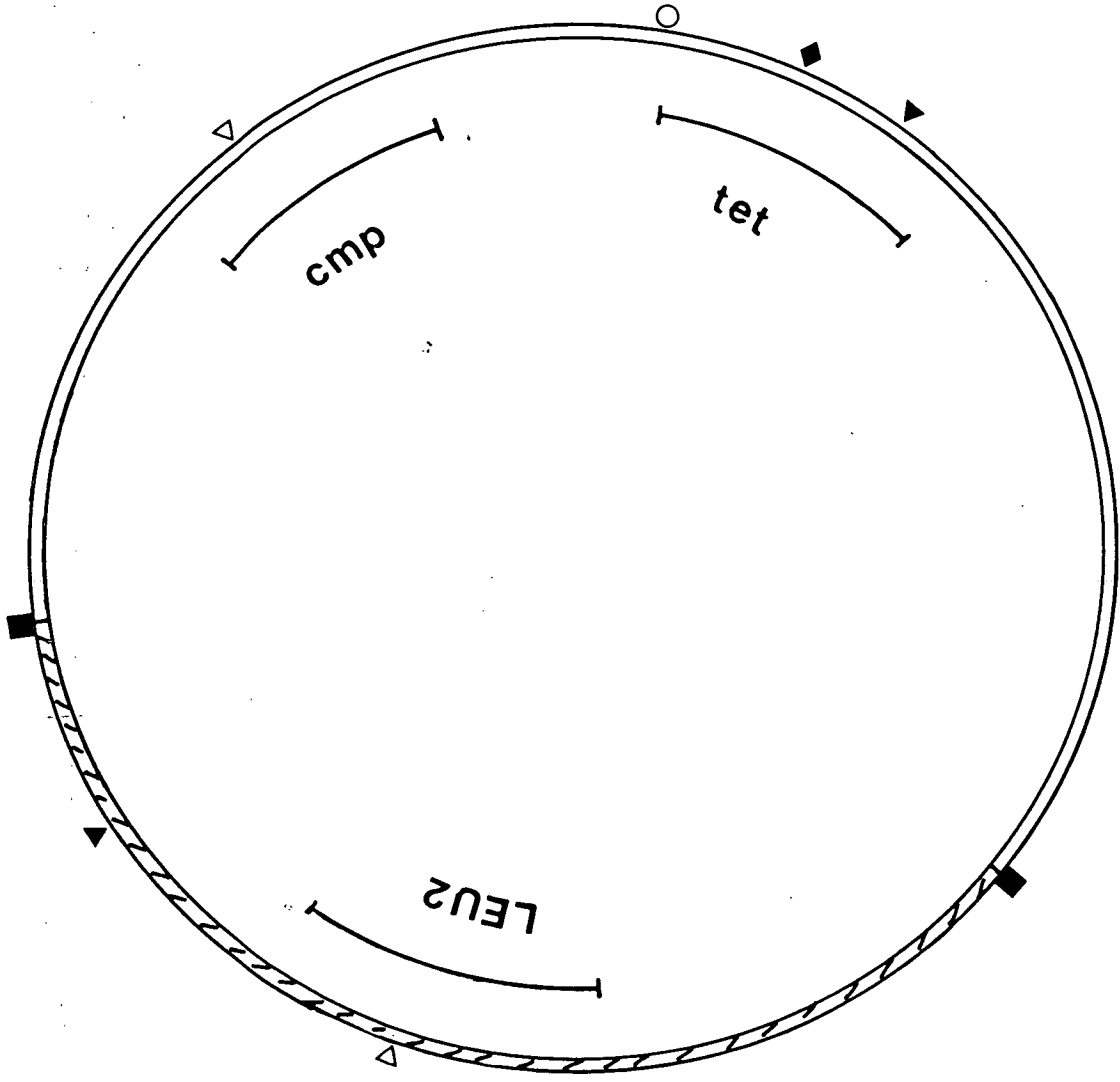







- | | | | |
|---|---|---|-----------------|
|  | Bacterial DNA sequences. |  | <u>Bam</u> HI |
|  | <i>S. cerevisiae</i> genomic DNA sequences. |  | <u>Eco</u> RI |
|  | 2µm plasmid DNA sequences. |  | <u>Hind</u> III |
| tet | Tetracycline resistance. |  | <u>Sal</u> I |
| cl_λ | λ repressor DNA sequence. | | |
| LEU2 | <i>S. cerevisiae</i> <u>LEU2</u> gene. | | |

Figure 2.3 Restriction map of pDAM6.



Size: 10.1Kb

-  Bacterial DNA sequences.
-  *S.cerevisiae* genomic DNA.
- cmp** Chloramphenicol resistance.
- tet** Tetracycline resistance.
- LEU2** *S.cerevisiae* LEU2 gene.

-  BamHI
-  EcoRI
-  HindIII
-  PstI
-  SalI

from the S. cerevisiae vector YEpl3 (Broach et al, 1979) containing the S. cerevisiae Leu 2⁺ gene into the PstI site in the ampicillin gene of bacterial plasmid pBR325. (Bolivar, 1978; P. Nurse, Personal Communication). It is capable of autonomous replication in E. coli but not in S. pombe. The plasmid codes for the antibiotic resistance genes to chloramphenicol and tetracycline for selection in E. coli.

pESPI was constructed from pDAM6 by deleting the SalI site from the PstI fragment containing the S. cerevisiae Leu 2⁺ gene. (P. Fantes, Personal Communication). This vector therefore contains a single SalI site in the tetracycline resistance gene. A restriction map of pDAM6 is shown in Figure 2.3.

(ii) Transformation of S. pombe

Essentially, the same protocol as Beach and Nurse, (1982), was used.

Cells were grown at 25°C to an optical density of 0.3 at 595 nm as measured on a Unicam SP600 spectrophotometer in 200 mls of EMM containing 0.5% glucose. The cells were harvested by centrifugation in sterile disposable McCartney bottles (Sterilin) in a MSE benchtop centrifuge at 3/4 speed for 5 minutes. The pellet was washed in 1.2 M Sorbitol, 20 mM citrate phosphate buffer pH 5.6, 40 mM EDTA and 150 mM β -mercaptoethanol (Sigma). After recentrifugation the cells were resuspended in 5 mls of 1.2 M Sorbitol, 50 mM citrate phosphate buffer pH 5.6, 30 mM β -mercaptoethanol and 5 mg/ml Novo SP24 enzyme (Novo enzymes) for protoplast formation. The cells were incubated in a shaking bath at 30°C. Periodically, a sample was observed under the microscope. When approximately 70% of the protoplasts had become spherical, the protoplasts were harvested. This usually took about 30

minutes. They were then washed three times in 25 mls of 1.2 M Sorbitol, 10 mM Tris-HCl pH7.6, gently resuspending the protoplasts with a Pasteur pipette between each wash. The protoplasts were centrifuged at $\frac{1}{2}$ speed in a MSE bench centrifuge for 2.5 minutes between each wash. The protoplasts were counted on a haemocytometer (Gelman Hawksley Ltd) and 3×10^7 aliquotted into 10 ml sterile disposable test tubes (Sterilin). The protoplasts were then centrifuged and resuspended in 100 μ l of 1.2 M Sorbitol, 10 mM Tris-HCl pH7.6 and 10 mM CaCl_2 . Plasmid DNA was added in less than 10 μ l bringing the final concentration to 10 - 20 μ g/ml. After incubation at 25°C for 15 minutes, 1 ml of 10 mM Tris-HCl pH7.6, 10 mM CaCl_2 , 20% (w/v) polyethylene glycol 4000 (BDH) was added to each tube and left for 15 minutes at 25°C. The cells were finally pelleted and resuspended in 200 ml of 10 mM Tris-HCl pH7.6, 10 mM CaCl_2 , 1.2 M Sorbitol, 0.5 mg/ml yeast extract and 5 mg/ml leucine and incubated at 30°C for 30 - 60 minutes before plating onto the surface of Osm - EMM selective plates. These plates were allowed to dry before incubating at 28°C.

(iii) Transformation of E. coli

The method of Dagart and Ehrlich (1979) was used with minor modifications.

A stationary phase L-Broth preculture of E. coli was diluted one in fifty into fresh L-Broth medium and incubated at 37°C with shaking. The cells were harvested when they had reached an optical density of 0.2 at 650 nm.

The culture was chilled for ten minutes on ice. The cells were

transferred to a sterile disposable McCartney (Sterilin) and centrifuged at maximum speed for 5 minutes on an MSE bench top centrifuge.

The pellet was resuspended in one half of the original culture volume of cold 0.1 M CaCl_2 and incubated at 0°C for 20 to 30 minutes. The cells were harvested as above and resuspended in 1/15 original culture volume of cold 0.1 M CaCl_2 . The cells were then dispensed in 100 μl aliquots into 10 ml sterile disposable test tubes to which plasmid DNA was added in less than 10 μl to give a final concentration of less than 1 $\mu\text{g}/\text{ml}$. This transformation mixture was incubated on ice for 10 to 30 minutes, followed by "heat shock" for 5 minutes at 37°C (or 2 minutes at 42°C).

The mixture was then returned to ice for a further 20 minutes. 2 mls of L-Broth was then added and the cells incubated at 37°C for 1 hour to allow the plasmid antibiotic resistance genes to express under non selective conditions. Finally, appropriate aliquots of cells were spread onto selective medium. These plates were allowed to dry and incubated at 37°C overnight.

4 Isolation of DNA and RNA

(i) General methods for handling DNA/RNA

(a) Extraction with phenol/chloroform

Proteins were removed routinely from a cell lysate mixture by extracting once with an equal volume of phenol, once with an equal volume of 1:1 mixture of phenol and chloroform, and once with chloroform. Chloroform was a 24:1 (v/v) mixture of chloroform and iso-amyl alcohol. Phenol was equilibrated with 0.1 M Tris-HCl pH 8.0 and containing 0.1% hydroxyquinoline and 0.2% β -mercaptoethanol. (Maniatis et al, 1982).

Extraction was carried out as follows. The aqueous mixture was mixed with an equal volume of phenol in a polypropylene tube. The contents were mixed until an emulsion formed. The tube was centrifuged at 1600 g for 3 minutes at room temperature to separate the phases. The aqueous phase was transferred to a fresh tube and the remaining organic phase discarded. An equal volume of 1:1 mixture of phenol and chloroform was used to extract the aqueous phase as before. The aqueous phase was extracted with an equal volume of chloroform.

The DNA was recovered by precipitation with ethanol.

(b) Ethanol precipitation

The volume of the DNA solution was estimated. The concentration of salt was adjusted with the stock solutions to that shown in Table 2.10 and mixed. Then 2 volumes of ice cold absolute ethanol was added, the solution mixed and incubated at -20°C for 30 minutes.

The DNA was pelleted by centrifugation either at 12000 g for 5 minutes or 5 minutes on an eppendorf microfuge. The supernatant was aspirated off and the pellet washed with 70% ethanol. The DNA was precipitated by centrifugation as before and the supernatant removed. The remaining traces of ethanol were removed by placing in a vacuum desiccator for 2 minutes.

The DNA pellet was dissolved in 20 mM Tris-HCl pH 7.6, 10 mM EDTA buffer.

When precipitating RNA the amount of absolute ethanol was increased to 2.5 volumes that of the original solution.

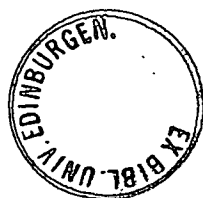


Table 2.10 Salt Solutions

Salt	Concentrated Solution	Final Solution
Sodium Acetate	2.5 M (pH 5.2)	0.25 M
Sodium Chloride	3.0 M	0.1 M
Ammonium Acetate	10.5 M	2.0 M

(ii) Isolation of nucleic acids from *S. pombe*(a) Rapid recovery of plasmid DNA from *S. pombe*

The method of Beach et al, 1982 was used. A yeast transformant colony was smeared onto a selective plate and incubated for 1 to 2 days. The cells were scraped off and resuspended in 0.5 mls of 20 mM Tris-HCl (pH 7.6), 20 mM EDTA; 1% Triton in a 15 ml glass boiling tube. To the suspension was added an equal volume of ballatini beads (BDH). The cells were mixed for 40 seconds on a vortex mixer and immediately phenol/chloroform extracted. The solution was decanted into a 1.5 ml sterile eppendorf tube and centrifuged for 1 minute on a Janetzki TH 12 eppendorf centrifuge. The supernatant was made 300 mM for Na⁺ with 3 M sodium acetate and precipitated with half the volume of cold (-20°C) isopropanol. After incubation at 4°C for 10 minutes the precipitate was pelleted for 5 minutes in an eppendorf centrifuge. The precipitate was resuspended in 100 µl of 10 mM Tris-HCl (pH 7.6), 1 mM EDTA (T.E.) buffer containing 5 µl of RNAase (1 mg/ml) and digested for 10 minutes at 65°C. The solution was phenol/chloroform extracted and centrifuged in an eppendorf centrifuge for 2 minutes.

The supernatant was made 0.1 M with 3 M NaCl and two volumes of 100% ethanol added. The sample was left at -20°C to precipitate the DNA. The DNA was pelleted in an eppendorf centrifuge for 3 minutes and washed with cold (-20°C) 70% ethanol. The suspension was recentrifuged and the DNA precipitate vacuum dried and resuspended in 50 µl of T.E. buffer.

(b) Large scale preparation of total *S. pombe* DNA

Essentially, the same method as Beach et al., (1982) was used. One litre of cells were grown in YEPD to an optical density of greater than 1 at 595 nm.

The cells were harvested by centrifugation at 5000 g for 5 minutes and resuspended in 60 mls of 1.2 M Sorbitol, 20 mM citrate phosphate buffer pH 5.6, 40 mM EDTA, 30 mM β -mercaptoethanol. Cells were then pelleted at 5000 g for 5 minutes and resuspended in 20 mls of the same buffer plus 5 mg/ml Novo SP234 to digest away the cell wall. The suspension was incubated at 37°C and tested periodically (approx. every 10 minutes) for greater than 70% lysis when the lysis buffer was added.

The protoplasts were lysed in 60 mls of 50 mM Tris-HCl (pH 7.6), 50 mM EDTA, 2% SDS at 65°C for 2 minutes. The lysate was then cooled to room temperature and phenol extracted. The mixture was centrifuged at 5000 g for 5 minutes and the aqueous (top) layer was phenol/chloroform extracted. This mixture was centrifuged at 5000 g for 5 minutes and the aqueous layer removed. To this was added 1/10 volume of 3 M sodium acetate followed by $\frac{1}{2}$ volume of cold (-20°C) isopropanol. The solution was incubated at 4°C for at least 30 minutes to allow the DNA to precipitate.

The precipitate was pelleted by centrifugation, resuspended in 6 mls T.E. mixed with 6.4 g of caesium chloride and 0.6 ml of 10 mg/ml solution of ethidium bromide.

The solution was spun in an MSE vertical rotor at 45 K for 24 hrs at room temperature on an MSE prepspin 50 ultracentrifuge. One clear band formed when illuminated with uv light which was removed with a syringe. To remove the ethidium bromide and CsCl $2\frac{1}{2}$ volumes of 70% ethanol were added and incubated at 4°C overnight. The DNA was pelleted

and resuspended in T.E. buffer. Two further ethanol precipitations were carried out to remove all the CsCl and ethidium.

(c) Rapid method of obtaining *S. pombe* total DNA

A scaled down version of the large scale DNA preparation for 100 mls of culture was used except that the cell wall digestion was carried out in 5 mls instead of 2 mls. All other volumes were scaled down by 1/10 of the large scale preparation.

After isopropanol precipitation of DNA, the pellet was resuspended in 2 mls of T.E. buffer pH 7.6 to which was added 60 μ l of 5 M NaCl and 150 μ l of RNAase (1 mg/ml). The solution was incubated at 65°C for 15 minutes. Then 220 μ l of proteinase K (100 ug/ml) was added and the preparation incubated at 45°C for 1 hour.

The mixture was phenol/chloroform extracted to remove all the proteinase K. After spinning at 5000 g for 5 minutes the aqueous layer was ethanol precipitated twice, vacuum dried and resuspended in 1 ml of T.E. buffer pH 7.6. The DNA concentration was estimated against known standards after electrophoresis through a 0.6% agarose gel.

(d) Isolation of total RNA from *S. pombe*

The cells were grown overnight in 50 mls of YEPD at 25°C to an optical density of 0.5 at 595 nm. Cells were harvested by centrifuging in a MSE bench top centrifuge at 5000 g for 5 minutes in sterile McCartney bottles. The pellet was resuspended in 1 ml of 0.1 M NaCl, 50 mM Tris-HCl pH 7.5, 2% Tri-iso-propyl-naphthalene - sulphonic acid (Eastman) T.N.S. and transferred to 15 ml glass tubes. An equal volume

of ballatini glass beads (acid washed) and phenol were then added. This mixture was vortexed twice for 40 seconds with 60 seconds on ice in between.

The cell lysate was immediately transferred to sterile 15 ml corex tubes (Dupont) and centrifuged at 10,000 g on angled Sorvall type SS34 rotar on a Sorvall RC-5B centrifuge. The aqueous layer was transferred to a 15 ml corex tube and extracted with an equal volume of chloroform. The RNA in the aqueous phase was precipitated in 0.1 M NaCl and 2.5 to 3 volumes of 100% ethanol at -20°C for at least 30 minutes.

The RNA was pelleted by centrifuging as above. The supernatant was aspirated off and the pellet vacuum dried. The pellet was resuspended in 500 μl of T.E. buffer pH 7.6.

The concentration of RNA was determined by the optical density at 260 nm on a Pye Unicam SP800 UV spectrophotometer.

(iii) Isolation of plasmid DNA from E. coli

(a) Rapid isolation of plasmid DNA from E. coli

Two methods were used depending on how much plasmid DNA was required:

I Boiling method of Holmes and Quigley, 1981.

II Alkaline lysis method of Birnboim and Doly, 1979.

I Boiling method.

When small amounts of plasmid DNA was required for analysis, that is approximately 1 μg , this was the method of choice as it was quicker than method 2.

Cells were inoculated into 5 mls of L-Broth medium plus the appropriate selective antibiotic in 10 ml sterile disposable test tubes and incubated overnight at 37°C. These cells were centrifuged at maximum speed on a MSE bench top centrifuge. The pellet was suspended in 150 µl of lysis buffer, 8% Sucrose, 5% Triton X-100 (BDH), 50 mM EDTA, 50 mM Tris-HCl (pH8) and transferred to a sterile 1.5 ml eppendorf tube. Lysozyme (Sigma) was added to a concentration of 1 mg/ml and the preparation incubated on ice for 5 minutes.

The sample was placed in a boiling water bath for forty seconds and immediately centrifuged for 10 minutes in an eppendorf microfuge. The gelatinous pellet that formed was removed with a sterile toothpick. The plasmid DNA was precipitated from the remaining supernatant by adding 1 volume of isopropanol and incubating at -20°C for 5 minutes.

The DNA was pelleted by centrifugation in an eppendorf microfuge for 5 minutes and the pellet resuspended in 100 µl of T.E. buffer pH 7.6 containing 0.1 M NaCl. The plasmid DNA was ethanol precipitated at -20°C, centrifuged in a microfuge for 10 minutes, the supernatant removed and the pellet dried in a vacuum desiccator. The DNA pellet was resuspended in 50 µl of T.E. buffer pH 7.6. Approximately 1 to 1.5 µg of plasmid DNA was routinely obtained by this method.

II Alkaline lysis

When more DNA was required a scaled up Birnboim and Doly preparation was used as the boiling method proved difficult to scale up.

50 mls of L-Broth plus the appropriate antibiotic was inoculated and grown up to stationary phase overnight. The cells were harvested by centrifugation in disposable McCartney bottles at maximum speed on a

MSE bench centrifuge. The pellet was resuspended in 5mls of 50 mM Glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8), lysozyme 2 mg/ml and transferred to a 30 ml corex tube. The preparation was incubated on ice for 5 minutes after which 10 mls of 0.2 M NaOH, 1% SDS was added and the mixture incubated on ice for a further 5 minutes.

The sample was neutralised using 7.5 mls of 3 M Na Acetate (pH 4.3) and left on ice at least 60 minutes. The preparation was centrifuged on a Sorvall angle SS34 rotar on a Sorvall RC-5B centrifuge at 7000 g for 5 minutes at 4°C. The supernatant was phenol extracted and centrifuged as above for 5 minutes.

The aqueous phase was removed and RNAase added to 50 µg/ml. The preparation was incubated at 65°C for 10 minutes followed by phenol/chloroform extraction. After centrifugation as before the aqueous phase was ethanol precipitated at -20°C. The DNA was pelleted at 7000 g for 5 minutes and the pellet washed with cold (-20°C) 70% ethanol. Finally, the DNA was centrifuged as above, the pellet dried in a vacuum desiccator and resuspended in 500 µl of T.E. buffer pH 7.6.

To estimate the DNA concentration, dilutions were run against known standards through a 0.6% agarose gel.

Approximately 100 µg were routinely obtained by this method.

(b) Large scale preparation of plasmid from E. coli

One litre of cells were grown in L-Broth plus selectable antibiotic at 37°C overnight to stationary phase. The cells were harvested by centrifugation in a Sorvall type GSA angle rotor at 7000 g for 5 minutes on a Sorvall RC-5B centrifuge. The cell pellet was resuspended in 40 mls of 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mM glucose and

4 mgs/ml lysozyme and incubated at room temperature for ten minutes. Then 80 mls of 0.2 M NaOH, 1% SDS (Sigma) was added, mixed and incubated on ice for five to ten minutes. To this, 40 mls of cold (0°C) 5 M potassium acetate pH 4.8 were added. (The potassium acetate was made as follows: to 60 mls of 5 M potassium acetate add 11.5 mls of glacial acetic acid and 28.5 mls of H₂O) and the contents mixed by inverting the tube sharply. The preparation was incubated on ice for one hour.

After this time 10 mls of H₂O was added, the contents mixed and spun at 3000 g for 5 minutes. The supernatant was poured through a plastic tea strainer to remove any floating lumps. To the supernatant was added 0.6 volume of isopropanol, the preparation mixed and left to stand at room temperature for 10 minutes. The nucleic acids were recovered by centrifugation at 8000 g for 5 minutes.

The pellet was resuspended in 24 mls of T.E. buffer pH 7.6 and 1.5 mls of 0.2 M EDTA (pH 8.0). The preparation was neutralised to pH 7.0 with 2 M Tris-base and the volume made up accurately to 28 mls with T.E. buffer pH 7.6. 31 g of Caesium chloride and 3 mls of ethidium bromide (10 mg/ml) was dissolved in the preparation to give a final density of approximately 1.59. The mixture was transferred to 40 ml polycarbonate MSE tubes and loaded onto a 8 x 40 MSE titanium vertical rotor. The rotor was spun in a MSE prespin ultracentrifuge at 40,000 g at 20°C for 24 hours.

The closed circular plasmid band was visible in the centre of the gradient when illuminated by uv light. The DNA was obtained by inserting a syringe through the wall of the centrifuge tube and collecting the plasmid band.

To remove the Caesium chloride and ethidium bromide from the DNA 3 volumes of 70% ethanol was added and the preparation incubated at 4°C

overnight. The DNA was pelleted and subjected to two further ethanol precipitations to remove all the ethidium bromide.

The final DNA pellet was dried under vacuum and the DNA concentration determined by the absorbance at 260 nm as read on a Pye Unicam UV spectrophotometer.

5 Molecular analysis of DNA and RNA

(i) Restriction digest analysis

Restriction enzymes were mostly obtained from BRL and used according to the manufacturer's instructions. Reactions typically contained 0.4 to 1 µg of DNA in a volume of 20 µl and were usually incubated at the recommended temperature for 1 hour.

When DNA was cut with two or more restriction enzymes the digestions were carried out simultaneously in the same buffer if both enzymes functioned at the same salt concentration. Alternatively the enzyme that functioned in the buffer of lower salt concentration was used first. Then the appropriate amount of salt plus the second enzyme was added and the incubation continued. Digests were terminated by the addition of 0.1 x the volume of 0.2 M EDTA pH 8 and/or heat inactivation at 65°C for 10 minutes.

(ii) Ligations

Ligation of cohesive ends were carried out according to the manufacturer's instructions. The final concentration of DNA to be ligated was in excess of 100 µg/ml. Typically 1 µg of DNA was ethanol precipitated in an 1.5 ml eppendorf tube. To this was added 1 µl of 10 x ligation buffer (0.2 M Tris-HCl pH 7.6; 0.1 M MgCl₂; 0.1 M

dithioerythritol), 1 μ l of gelatin solution (1 mg/ml) and 1 μ l of ATP solution (10 mM). Approximately 1 unit of T_4 DNA ligase (Boehringer) was added and the mixture incubated at 12°C overnight. The reaction was terminated by the addition of 0.1 x volume 0.2 M EDTA (pH 8). The ligation mixture was incubated at 70°C for 10 minutes before using for bacterial transformations.

(iii) Agarose gel electrophoresis

The vertical gel system used routinely was described by Southern (1979). The gel dimensions were 140 mm in width, 100 mm in depth and 3 mm thick. The slot dimensions were 9 mm in depth, 5 mm in width and 3 mm thick. Unless otherwise stated electrophoresis was through 0.6% agarose (type II, low E.E.O. (Sigma)) dissolved in electrophoresis buffer (36 mM Tris-HCl (pH 7.7), 30 mM NaH_2PO_4 ; 1 mM EDTA). The buffer was stored as a x10 stock at 4°C. The applied voltage was no more than 5V/cm. Following electrophoresis the gel was stained in a 0.5 μ g/ml solution of ethidium bromide (Sigma). The DNA fragments were detected by illumination from a UV transilluminator (Ultra Violet Products Ltd) and photographs obtained using a Polaroid MP4 land camera with Polaroid Type 667 film.

A horizontal gel apparatus was used to recover a specific DNA fragment from an agarose gel. The gel dimensions were 170 mm in length; 150 mm in width and 10 mm deep. The slot dimensions were 1 mm in length; 5 mm in width and 8 mm in depth.

The DNA samples were mixed with gel loading buffer which contained 5% glycerol and 0.025% bromophenol blue added from a x10 stock.

(iv) Recovery of DNA from agarose gels

For some subcloning of DNA fragments the method used was to remove a defined restriction fragment by cutting it out of a horizontal low melting point 1.2% agarose gel (Sigma). The method used was that given in Maniatis et al, 1982.

The low melting agarose was dissolved in electrophoresis buffer by heating in a microwave oven for 5 minutes. This was allowed to cool, ethidium bromide added to a final concentration of 0.5 µg/ml and the gel poured at 4°C. The gel was run for 8 hours at 5V/cm.

The gel was checked under UV to make sure that the desired DNA fragment had separated. Gel including the fragment was cut out using a scalpel and placed in a 1.5 ml eppendorf tube. The volume of the gel was estimated and x 5 volume 20 mM Tris-HCl (pH 8) 1 mM EDTA added. The sample was heated at 65°C for 5 minutes and cooled for 10 minutes to allow the preparation to reach room temperature.

The melted gel slice was phenol extracted. The aqueous phase was recovered after spinning for 1 minute in a microfuge. This aqueous phase was phenol/chloroform extracted and then chloroform extracted. The DNA was recovered from the final aqueous layer by ethanol precipitation.

It was found that DNA recovered by this method would not reliably ligate. The preparation was further purified by chromatography on a NACS-52 column (BRL). The column was constructed from a 1 ml disposable syringe plugged with siliconized glass wool. One ml of NACS-52 suspension (prepared according to the manufacturer's recommendations) was loaded onto the column and allowed to drain under gravity flow. The column was washed with 5 mls of 0.5 M NaCl and allowed to drain

completely. The DNA preparation was dissolved in 0.5 M NaCl and loaded onto the column. The column was washed with 5 mls of 0.5 M NaCl and allowed to drain. To reduce the dilution of recovered DNA, residual 0.5 M NaCl in the column was removed by air pressure from the syringe piston. The DNA was eluted from the column with 0.3 mls of 2 M NaCl. The DNA was collected and purified by ethanol precipitation.

(v) Nick translation of DNA

Radioactive ^{32}P labelled DNA probes were prepared by nick translation using the method of Rigby et al, 1977. About 1 μg of DNA was mixed with 20 μCi (2 μl) of $\alpha\text{-}^{32}\text{P}$ -dTTP (410 Ci/mmol, Amersham), 5 μl of each of the other unlabelled nucleotides (from 200 μM stock solutions), 5 μl of 10 x nick translation buffer (1 x nick translation buffer: 50 mM Tris-HCl (pH 7.8), 10 μM β -mercaptoethanol, 5 mM Mg Cl_2 , 100 $\mu\text{g/ml}$ BSA) and 25 μl of H_2O .

To start the reaction 1 μl of a 2×10^4 fold dilution (in H_2O) of DNAase I (BRL) stock solution (1 mg/ml in 1 x nick translation buffer containing 50% (v/v) glycerol) and 2 μl (10 units) of DNA polymerase I (Amersham) was added to the nick translation mixture. The reaction mixture was incubated at 15°C .

The incorporation of ^{32}P -dTTP into the DNA was monitored from 1 μl samples at specific times after the start of the reaction. Samples were spotted onto Whatman GF/C filters and the radioactivity measured by the Chernokov method in the ^3H channel of a Hewlett Packard model 2425 liquid scintillation counter. The filter was then washed three times in 100 mls of cold 5% trichloroacetic acid (TCA). The filter was dried on a hot plate and the radioactivity measured as

before. The two values were used to calculate the % incorporation of radioactivity into the DNA.

The reaction was terminated by the addition of 1/5 volume of 0.2 M EDTA (pH 8). Immediately before use as a probe in hybridisation experiments, the radioactive DNA was denatured by boiling for 5 - 10 minutes.

(vi) Hybridisation of Nucleic Acids

(a) Southern Transfer

Southern transfer (Southern, 1975) was used to detect specific sequences of DNA within a particular cloned or genomic DNA digest. In outline, DNA fragments that have been separated according to size by electrophoresis through an agarose gel are denatured, transferred to a nitrocellulose filter and immobilised. The transfer is carried out by setting up a flow of liquid through the gel and the nitrocellulose filter so that DNA fragments are eluted from the gel and bind to the nitrocellulose paper. The DNA attached to the filter is then hybridised to ³²P-labelled DNA or RNA and autoradiography used to locate the radioactive probe.

The conditions and apparatus used were similar to Southern, 1975 as modified by Maniatis et al, (1981). The DNA in the agarose gel was soaked in 0.25 M HCl for 15 minutes. The acid partially hydrolyses the DNA causing depurination which aids the transfer of large DNA fragments (Wahl et al, 1979). The acid was decanted off and the gel immersed in 1.5 M NaCl and 0.5 M NaOH for 15 to 30 minutes to denature the DNA causing strand separation. The alkali solution was drawn off and the gel washed with distilled water. The gel was then soaked in a

neutralising solution of 3 M sodium acetate pH 5.5 for 30 minutes.

The gel was then placed on sheets of chromatography paper soaked in 20 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate) on top of a plastic tray. A cellulose nitrate filter (Schleicher and Schuell BA 85) was cut to the size of the gel, presoaked in 20 x SSC and placed over the gel taking care not to trap any air bubbles. Two pieces of chromatography paper were soaked in 20 x SSC and laid over the top of the filter, again making sure there were no air bubbles. Paper towels were then stacked on top and weighed down with glass bottles. To prevent "short circuiting" of the liquid path between the tray and the paper towels, old X-ray films were used to form a water tight border round the gel.

DNA transfer was carried out overnight. The filter was labelled gel side up with the positions of the gel slots using a ball point pen. The filter was blotted between two sheets of chromatography paper and baked for 2 hours in a vacuum oven at 80°C. The filter was stored until use in a desiccator at room temperature.

(b) Hybridisation of filter

The filter was prehybridised in 30 mls of prehybridisation fluid (2 x SSC, 0.5% SDS, 2 x Denhardt's solution (see below), 50 µg/ml denatured salmon sperm DNA (see below)), contained in a glass staining jar. Prehybridisation was carried out for 3 to 4 hours at 60°C.

Hybridisation was carried out in 30 mls of the same buffer as above. Denatured probe (see nick translation) was added and liquid paraffin poured on top to prevent loss of hybridisation mixture by evaporation. The glass staining jar was sealed with masking tape and

incubated at 70°C overnight.

After hybridisation, the filter was washed three times with 1 x SSC at 60°C for 1 hour. The filter was dried by blotting with chromatography paper, wrapped in Saran wrap, and applied to an X-ray film to obtain an autoradiographic image.

Denhardt's solution (50 x) per 500 ml of H₂O:

Ficoll (Sigma) 5.0 g

polyvinylpyrrolidone (Sigma) 5.0 g

BSA (Sigma) 5.0 g

Denatured salmon sperm DNA:

Salmon sperm DNA (Sigma) was dissolved in H₂O at a concentration of 10 mg/ml and denatured by incubating in a boiling water bath for 10 minutes.

(c) Autoradiography

The filter was taped to chromatography paper and placed in a X-ray film cassette. In a dark room a sheet of preflashed Kodak X-Omat S type 1 X-ray film was placed on top of the filter. An intensifier screen (Du Pont Cromex - Lighting Plus) was placed next to the X-ray film. The cassette was sealed and placed at -70°C to expose the film.

The film was developed in Kodak LX -24 developer for 5 minutes, washed once in tap water and fixed for 5 minutes in Kodak FX - 40 fixer.

The film was then rinsed extensively in tap water.

(vii) Hybridisation of RNA

In outline, total RNA was separated on a formaldehyde denaturing agarose gel (Goldberg, 1980) and blotted to nitrocellulose using the method of Thomas, 1980.

Subsequent operations to locate the RNA transcript of interest were as for DNA blots.

(a) Gel electrophoresis of RNA

A vertical gel apparatus of dimensions 20 cm wide by 20 cm deep and 0.5 cm thick was used. Sample slots were 80 mm wide and 1 cm deep. The bottom of the gel was sealed with 1.5% agarose dissolved in 1 x gel buffer (25 mM MOPS (Sigma) pH 7, 5 mM sodium acetate, 1 mM EDTA). Then 1.2% agarose was dissolved in 1 x gel buffer to 5/6 of the final volume. The dissolved agarose was cooled to 50-60°C in a water bath and 1/6 volume of 38% formaldehyde solution (BDH) added and the gel poured. After the gel had set, the gel tank was filled with enough 1 x gel buffer to cover the electrodes. During electrophoresis the buffer was found to leak from the gel apparatus and therefore a peristaltic pump was used to recirculate the buffer.

(b) Preparation of the RNA samples

40 µg of S. pombe RNA was ethanol precipitated using 2.5 x volumes of absolute ethanol. The precipitate was dried in a vacuum desiccator and dissolved in 100 µl of loading mixture (50% v/v formamide (BDH),

1/6 volume of 38% formaldehyde solution, made up to final volume with 1 x gel buffer). The sample was incubated for 5 minutes at 60°C to denature the RNA and 2 µl of 2 mg/ml ethidium bromide solution added. The samples were loaded and electrophoresis continued overnight at 50 v. The gel was placed on top of a UV transilluminator and photographed as for a DNA gel. For the RNA gels described in this work, the S. pombe ribosomal RNA bands were used as molecular weight markers. Therefore, to calibrate the picture of the gel with the final autoradiograph, a ruler was included in the photograph to determine the distance that the ribosomal bands had travelled from the top of the gel.

(c) Northern Transfer

Two methods were used depending on whether nitrocellulose or gene-screen were used to bind the RNA.

I Binding RNA to Nitrocellulose

The nitrocellulose filter was soaked for 30 minutes before use in 20 x SSC. Essentially the same method was used as for Southern transfer except that the gel required no pretreatment and was placed directly onto the chromatography sheets. The gel was blotted overnight. The transfer of RNA to the nitrocellulose filter was checked under UV light. The filter was dried and baked in a vacuum oven at 80°C for 3 to 4 hours. The filter was prehybridised in 30 mls of prehybridisation solution (50% formamide, 5 x SSC, 50 mM Na PO₄ buffer pH 6.5, 2 x Denhardt's solution, 50 µg/ml denatured Salmon sperm DNA) and incubated at 42°C overnight in a glass staining jar.

Denatured radioactive probe was then added to the prehybridisation solution. Paraffin was added as an overlayer to prevent the filter

drying out. The mixture was incubated at 42°C overnight.

Four changes of 2 x SSC, 0.1% SDS at room temperature for at least 5 minutes each was used to wash the filter. This was followed by two washes in 0.1 x SSC, 0.1% SDS at 50°C for at least 15 minutes.

The filter was then dried and autoradiographed as described for DNA hybridisations.

II Binding RNA to Genescreen Nylon Membrane

Genescreen (New England Nuclear) was used as recommended by the manufacturers. The genescreen membrane was soaked before use in 0.025M Na₂ HPO₄/Na H₂ PO₄ (pH 6.5) for 15 to 20 minutes. RNA transfer was carried out as for the nitrocellulose filter except that the transfer buffer was 0.025 M Na₂ HPO₄/Na H₂ PO₄ (pH 6.5). The filter was dried and baked in a vacuum oven at 80°C for 2 to 4 hours.

The filter was prehybridised in a 10 ml prehybridisation solution (50% formamide, 0.2% polyvinyl-pyrrolidone M. W. 40,000 [Sigma], 0.2% bovine serumalbumin [Sigma], 0.2% ficoll M. W. 400,000 [Sigma], 0.05 M Tris-HCl pH 7.5, 0.1% sodium pyrophosphate, 1.0% SDS, 10% dextran sulphate M. W. 500,000 (Pharmacia) and 100 µg ml⁻¹ denatured salmon sperm DNA). The solution was added to a sealable plastic bag containing the membrane filter, the bag sealed and incubated at 42°C for 6 hours.

Denatured radioactive probe was added in 3 mls of the prehybridisation solution (minus dextran sulphate) to the bag containing the membrane. The plastic bag was then resealed and incubated at 42°C for 16 to 24 hours.

The membrane was then washed twice with 100 mls of 2 x SSC at room temperature for 5 minutes. This was followed by two washes in 100 mls of 2 x SSC and 1.0% SDS at 65°C for 30 minutes with constant

agitation. Finally, the membrane was washed twice in 100 mls of 0.1 x SSC at room temperature for 30 minutes with constant agitation.

The membrane was then dried and autoradiographed as before.

Genescreen was used when a filter was expected to be reprobred with a different radioactive probe. Nitrocellulose was found to be unsuitable for reprobing as it was fragile and cracked easily during the rehybridisation procedure.

The following protocol was used to remove the labelled probe from a genescreen membrane. The filter was washed in 250 mls of rehybridisation solution (0.005 M Tris-HCl pH 8.0, 0.0002M Na₂ EDTA, 0.05% Sodium Pyrophosphate, 0.002% polyvinyl - pyrrolidone M. W. 40,000, 0.002% bovine serum albumin, 0.002% ficoll M. W. 400,000) and incubated with constant agitation for 1 to 3 hours at 65 to 70°C.

After incubation the membrane was air dried and treated as before.

CHAPTER 3 Isolation of DNA Sequences that Complement a cdc 22 Mutation1 Introduction

The method used for isolating the cdc 22 gene was essentially the same as that developed to clone cdc genes from S. cerevisiae. (Nasmyth and Reed, 1980; Clarke and Carbon, 1980). From a pool of wild type yeast DNA fragments carried in a plasmid "gene library" those that were able to complement the temperature sensitive cdc 22-M45 mutation were selected after transformation experiments. The rationale for the cloning experiment is shown in figure 3.1.

2 Gene libraries

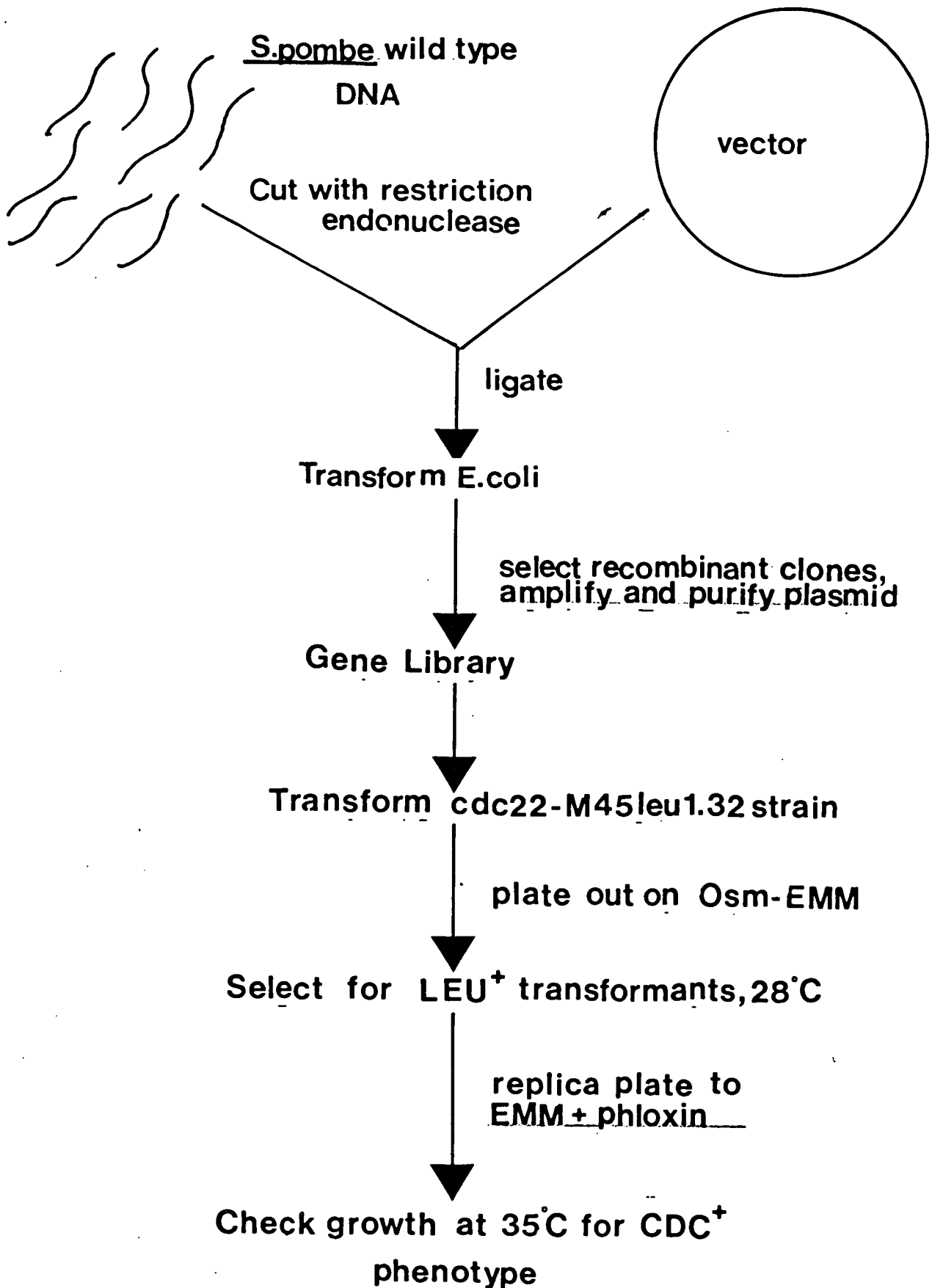
Three independently constructed gene libraries were used in the transformation experiments.

The first, GB1, was constructed by Dr P. A. Fantes and Dr J. Ogden from a partial Sau3A digestion of genomic wild type S. pombe DNA. As Sau3A leaves the same GATC cohesive ends as the restriction enzyme BamHI the fragments could be inserted into the BamHI site of the tetracycline resistance gene of the vector pDB248 (Beach and Nurse, 1980).

This DNA was used to transform the E. coli strain JA221 and 10,000 independent ampicillin resistant, tetracycline sensitive colonies were picked to make the gene library. The average insert size was estimated from ten independent colonies and found to be approximately 4 kb. (Ogden, J., Personal Communication).

The other two gene libraries were obtained from Dr Paul Nurse. The first GB2 was a partial HindIII digest of wild type S. pombe DNA ligated into the vector pDB262 (see Materials and Methods). The gene bank was constructed from 20,000 E. coli independent recombinants.

Figure 3.1 Rationale for Cloning Experiments



The second, GB3, was a total HindIII digest into the same vector. The gene library was made from 30,000 E. coli clones.

As only a small amount of GB2 and GB3 was available, 10 µg of each gene library, they were first amplified by retransforming E. coli. The 100,000 recombinant E. coli clones obtained were pooled and a large scale plasmid preparation made by the alkaline lysis method (see Materials and Methods) for each gene library.

A small amount of unamplified GB2 DNA remained which was also used in the transformation experiments.

3 Transformation of a cdc 22-M45 leu 1.32 strain with the gene library

Both the plasmid vectors used in the preparation of the gene libraries carried the leu 2⁺ gene of S. cerevisiae which complements the leu 1.32 mutation of S. pombe. Therefore a leu 1.32 mutation was crossed into a cdc 22-M45 strain to enable yeast transformants carrying a plasmid to be selected.

Transformation of the cdc 22-M45 leu 1.32 strain was as detailed in Materials and Methods.

Preliminary characterisation of the cdc 22-M45 mutation showed that it was osmotically suppressible on Osm EMM agar plates. Therefore, cdc⁺ leu⁺ transformants could not be directly selected. To solve this problem leu⁺ transformants were first selected at 28°C before testing for the cdc⁺ phenotype by replica plating to EMM plus phloxin at 35°C and scoring for growth.

The number of leu⁺ transformants screened together with the number of cdc⁺ leu⁺ colonies obtained for each gene library is shown in table 3.1.

The six cdc⁺ leu⁺ transformants from the GB2 unamplified gene

Table 3.1Transformation of cdc 22-M45 leu 1.32 with gene library DNA

<u>GENE BANK</u>	<u>NO OF <u>leu</u>⁺ TRANSFORMANTS</u>	<u>NO OF <u>cdc</u>⁺ TRANSFORMANTS</u>
GB1	36,000	10
GB2	21,000	0
GB3	19,000	0
GB2 unamplified	7,200	6

library all divided at wild type size at 35°C. Growth also appeared normal as colonies appeared after two days on EMM at 35°C. These transformants were termed cdc 22-GB2(1) to cdc 22-GB2(6).

The phenotypes of the ten cdc⁺ leu⁺ transformants obtained from the GB1 gene library were different from those described above. These transformants divided at two to three times the wild type size at 35°C. Colony growth was also slower than wild type as it took at least four days at 35°C on EMM for colonies to appear.

From table 3.1 it is interesting to observe that no cdc⁺ leu⁺ transformants were obtained from the GB2 gene library that had been amplified in E. coli though a greater number of leu⁺ transformants were tested for growth at 35°C. This suggests that plasmids that carried the cdc 22 gene had been selected against during amplification. The selection could have occurred during transformation and/or growth of the plasmids in E. coli (Hannahan, 1983). The method of plasmid isolation could also have imposed a selection (Maniatis et al, 1982).

4 Mitotic Stability and Cosegregation

S. pombe strains carrying the plasmids pDB248 or pDB262 are mitotically unstable when grown with or without selection. Plasmid is lost due to not possessing a mechanism for efficient segregation to mother and daughter at mitosis. Therefore a mitotic instability for cdc⁺ and leu⁺ when the transformants are grown under non-selective conditions would be characteristic of the genes determining these phenotypes being carried on the above vectors. If both cdc⁺ and leu⁺ were on the same plasmid cosegregation of these markers would be expected.

To test this, each of the cdc⁺ leu⁺ transformant strains were streaked out non selectively on YE agar at 25°C. Single colonies were picked onto a YE agar master plate and grown overnight at 25°C. They were then replica plated to EMM with or without leucine at 25°C and 35°C. The cdc and leu phenotype of each colony was determined and the results obtained are shown in table 3.2.

As can be seen from this table in the transformant strains cdc 22-GB2(1) to cdc 22-GB2(6) the cdc⁺ and leu⁺ markers were both mitotically unstable and cosegregated.

The transformant strains cdc 22-GB1(1) to cdc 22-GB1(10) were all mitotically unstable for cdc⁺ and leu⁺. However, in only two strains cdc 22-GB1(7) and cdc 22-GB1(10) did the two markers show cosegregation in nearly all cases. In the other eight transformants it was found that the two mitotically unstable markers behaved independently and the phenotypes could be separated from one another. This would suggest that two distinct plasmids were present in these yeast transformants, one carrying each marker.

5 Isolation of putative cdc 22⁺ containing plasmids

In order to isolate and amplify the plasmids DNA was obtained from the transformants by vortex mixing in the presence of glass beads to break the cells mechanically (See Materials and Methods). This DNA was used to transform the E. coli strain BJ5183.

Plasmids were obtained from transformant strains cdc 22-GB2(1) to cdc 22-GB2(4), cdc 22-GB1(7) and cdc 22-GB1(10) that were capable of retransforming a cdc 22-M45 leu 1.32 strain to give a cdc⁺ leu⁺ phenotype at approximately 10³ transformants per µg of DNA added. In each case a number of separate E. coli transformants from each yeast

Table 3.2 - Mitotic Stability of cdc^+ and leu^+ in cdc^+ leu^+ transformants

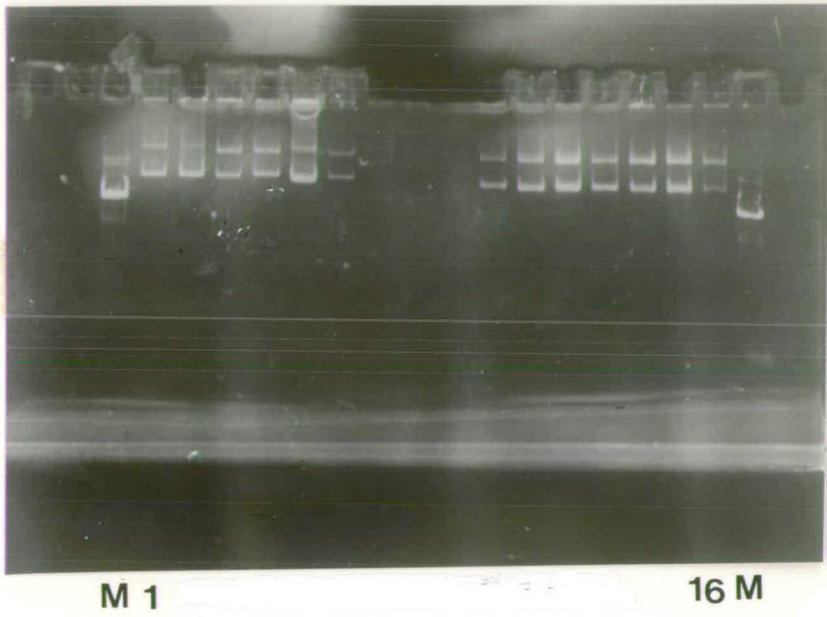
STRAIN	PHENOTYPE				TOTAL NO
	cdc^+ leu^+	cdc^+ leu^-	cdc^- leu^+	cdc^- leu^-	
<u>cdc</u> 22-GB1(1)	28	5	39	23	95
<u>cdc</u> 22-GB1(2)	31	46	14	9	100
<u>cdc</u> 22-GB1(3)	59	18	12	9	98
<u>cdc</u> 22-GB1(4)	34	8	13	11	63
<u>cdc</u> 22-GB1(5)	38	23	22	0	83
<u>cdc</u> 22-GB1(6)	3	23	9	0	35
<u>cdc</u> 22-GB1(7)	25	0	1	25	51
<u>cdc</u> 22-GB1(8)	8	6	22	12	42
<u>cdc</u> 22-GB1(9)	12	1	4	1	18
<u>cdc</u> 22-GB1(10)	37	0	2	11	50
<u>cdc</u> 22-GB2(1)	64	0	0	36	100
<u>cdc</u> 22-GB2(2)	55	0	0	43	98
<u>cdc</u> 22-GB2(3)	31	0	0	68	99
<u>cdc</u> 22-GB2(4)	57	0	0	40	97
<u>cdc</u> 22-GB2(5)	28	0	0	72	100
<u>cdc</u> 22-GB2(6)	48	0	0	52	100

transformant strain were screened for cdc⁺ function in cdc 22-M45 leu 1.32 before a plasmid capable of complementation of the cdc defect was isolated.

The gel shown in figure 3.2 illustrates this point. Out of a number of plasmids isolated from the same yeast transformant strain only a fraction were able to complement a cdc 22-M45 leu 1.32 strain. Plasmid DNA obtained from 16 separate E. coli clones obtained by transformation with DNA prepared from the yeast transformant strain cdc 22-GB2(2) were separated by electrophoresis on a 0.6% agarose gel. Only four of these plasmids (figure 3.2; tracks 11,12,14 and 16) complemented the cdc 22-M45 leu 1.32 strain.

The other plasmids could have arisen by cotransformation from the original gene bank as has been shown by Beach et al, (1982) or generated by recombination either in S. pombe and/or E. coli.

In the transformant strains that showed independent segregation of leu⁺ and cdc⁺, plasmids conferring a cdc⁻ leu⁺ phenotype on a cdc 22-M45 leu 1.32 strain were isolated in E. coli. None of the plasmids isolated conferred a leu⁻ phenotype as tested in the E. coli leucine auxotroph strain JA221. This suggests that no cdc⁺ leu⁻ plasmids could be isolated. Presumably no cdc⁺ leu⁻ plasmids were obtained because these plasmids were not capable of growth in E. coli due either to the absence of a functional origin of replication and/or not expressing the gene for ampicillin resistance selected for in the transformation experiments.

Figure 3.2

M = pDB262

Figure 3.2

Plasmid DNA was isolated from 16 separate E.coli clones that had been transformed with the same yeast transformant strain cdc 22 - GB2(2). The DNA was separated by electrophoresis on a 0.6% agarose gel at 50 V for 3 hours. Following electrophoresis the gel was stained in ethidium bromide. The DNA fragments were detected by fluorescence when illuminated by UV. The photograph was taken using a Polaroid MP4 land camera as described in Materials and Methods.

6 Transformation of cdc 22-C11 leu 1.32

During the course of this work Dr Jim Creanor isolated a number of additional S. pombe cdc mutations (J. Creanor, Personal Communication). One of these mutants was found to be a different allele of cdc 22 designated cdc 22-C11 (See Chapter 7).

A cdc 22-C11 leu 1.32 strain was constructed and transformed with each of the six cdc⁺ leu⁺ plasmids. All the leu⁺ transformants were cdc⁺ when tested for growth at 35°C.

7 Summary

The experiments described the isolation of sixteen cdc⁺ leu⁺ transformants from three separate gene libraries. All sixteen strains were mitotically unstable for leu⁺ and cdc⁺, indicating that the genes encoding these phenotypes were carried on autonomously replicating plasmids.

The transformants could be divided into two groups according to their mitotic segregation pattern:-

1. In eight of the transformants the cdc⁺ and leu⁺ markers cosegregated indicating that they were carried on the same plasmid.
2. The remaining eight showed independent segregation of cdc⁺ and leu⁺ suggesting that the markers were carried on two separate plasmids.

Plasmids were obtained in E. coli from six of the eight transformant strains (group 1, above) and these retransformed cdc 22-M45 leu 1.32 and cdc 22-C11 leu 1.32 strains at a high frequency.

CHAPTER 4 Characterisation of cdc 22 - M45 Complementing Plasmids

1 Introduction

A necessary first step after the isolation of a number of plasmids able to complement cdc 22 mutations was to determine which, if any, carried the same S. pombe DNA fragment.

Secondly, it was important to investigate whether the S. pombe cloned sequence present on each plasmid was the same as that originally present in the genome and was not the result of a ligation artifact caused during the construction of the gene library.

Finally, complementation of a mutation was not sufficient proof that the cloned sequences contained the desired gene as it could also code for an extragenic suppressor of the mutation (Bostein and Davis, 1981).

Each of the above points was investigated in the experiments described in this chapter.

2 Restriction Digest Analysis

To determine if the six cdc 22 - M45 complementing plasmids isolated in E. coli all contained the same S. pombe DNA fragment restriction digest analysis was carried out with each plasmid. Figure 4.1 (b) shows the vector pDB262 and plasmid extracted from the cdc 22 - GB2(1) yeast transformant strain digested with the restriction enzymes BamHI, EcoRI, HindIII or PstI and separated on a 0.6% agarose gel by electrophoresis (detailed in Materials and Methods). By plotting the log (molecular weight) of known markers (HindIII digested lambda DNA) against mobility through the gel, the size of the bands obtained could be estimated (Figure 4.1(a)).

Figure 4.1(a)

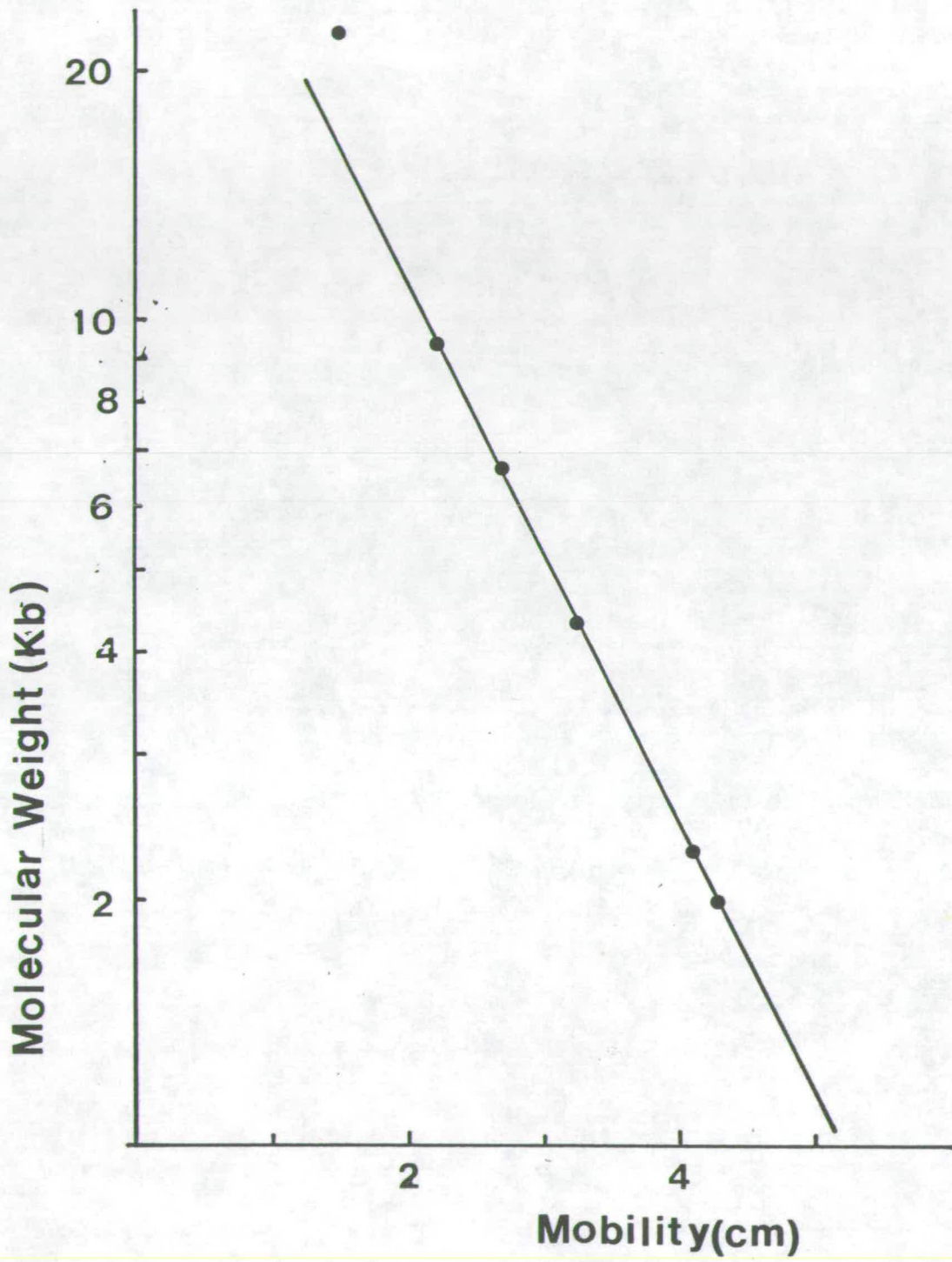


Figure 4.1(a) Size of DNA Fragments Obtained After Digestion of pSP22(1) with Different Restriction enzymes

A calibration curve of log (molecular weight) against mobility was plotted for the HindIII digested λ DNA fragments of size. This curve is shown in Figure 4.1(b). Using this curve the molecular weight of the bands obtained from the digestion of pDB262 and pSP22(1) could be estimated.

Lane 3	3 DNA fragments of sizes approximately 12.6 Kb, 2.1 Kb 1.1 Kb
Lane 4	1 fragment of size 10.6 Kb
Lane 5	3 fragments of sizes 10.6 Kb, 3.0 Kb, 2.6 Kb
Lane 6	1 band of size 10.6 Kb
Lane 7	2 bands 11.0 Kb, 4.9 Kb
Lane 8	1 band 10.6 Kb
Lane 9	4 bands 5.7 Kb, 4.6 Kb, 3.3 Kb, 2.1 Kb
Lane 10	2 bands 6.1 Kb, 4.6 Kb

This demonstrates that the S. pombe HindIII DNA fragment cloned in pDB262 contained 2 PstI sites, 1 HindIII site, 2 EcoRI sites, and 1 BamHI site.

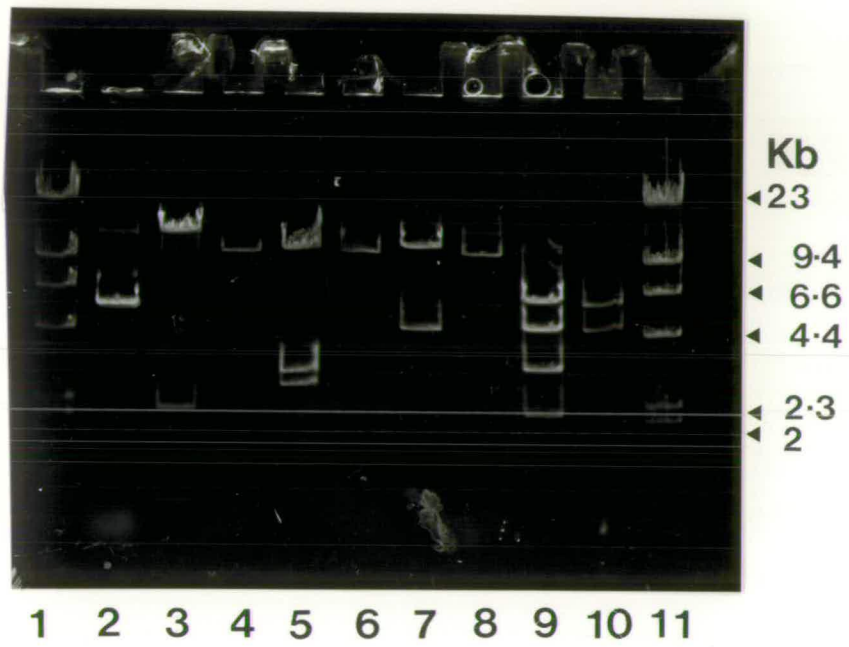
Figure 4.1(b)

Figure 4.1

(b) Restriction Digest Analysis of pDB262 and Plasmid Obtained from cdc 22 - M45 - GB2(1) (Termed pSP22(1))

Both pDB262 and pSP22(1) were digested to completion with BamHI, EcoRI, HindIII or PstI. The digested plasmid DNA was separated by electrophoresis through a 0.6% agarose gel at 50 V. Following electrophoresis the gel was stained in 200 mls of distilled water containing 0.5 µg/ml solution of ethidium bromide. The DNA fragments were detected by illumination from a UV transilluminator and photographed using a Polaroid MP4 land camera. The sizes of the different DNA fragments were estimated from molecular weight markers of known size (HindIII digested λ DNA; see Figure 4.1a)

- Lane 1 0.7 µg HindIII λ DNA; (Molecular weight markers)
- Lane 2 0.5 µg pDB262 undigested
- Lane 3 1 µg pSP22(1) cut PstI
- Lane 4 0.5 µg pDB262 cut PstI
- Lane 5 1 µg pSP22(1) cut HindIII
- Lane 6 0.5 µg pDB262 cut HindIII
- Lane 7 1 µg pSP22(1) cut BamHI
- Lane 8 0.5 µg pDB262 cut BamHI
- Lane 9 1 µg pSP22(1) cut EcoRI
- Lane 10 0.5 µg pDB262 cut EcoRI
- Lane 11 0.7 µg HindIII digested λ DNA

Further analysis with additional restriction enzymes or double digests involving two different restriction enzymes enabled a detailed restriction map to be constructed (Figure 4.2(a)). This data demonstrated that a 5.6 Kb S. pombe DNA fragment was present in the plasmid obtained from the cdc 22 - GB2(1) transformant. Similar investigations on plasmids isolated from the transformants cdc 22 - GB2(2) to cdc 22 - GB2(4) showed that the same 5.6 Kb fragment was present in each plasmid (Data not shown).

The plasmid obtained from a cdc 22 - GB1(7) strain was also subjected to restriction digest analysis. Figure 4.3 shows pDB248 and the plasmid isolated from a cdc 22 - GB2(7) transformant cut with the restriction enzymes EcoRI, HindIII or KpnI and separated by electrophoresis on a 0.6% agarose gel. From the information in Figure 4.3, plus further investigations, a restriction map was constructed (Figure 4.2(b)). This showed that the plasmid contained a 2.2 Kb S. pombe insert. Plasmid isolated from a cdc 22 - GB1(10) transformant also contained the same 2.2 Kb S. pombe DNA insert. If the two restriction maps presented in Figures 4.2(a) and 4.2(b) are compared it is clear that the 2.2 Kb insert is different from the 5.6 Kb fragment.

Therefore two different sequences had been isolated that complemented the cdc 22 - M45 mutation. The plasmids containing the 5.6 Kb fragment will now be referred to as pSP22(1) and the plasmids containing the 2.2 Kb fragment as pSP22(2).

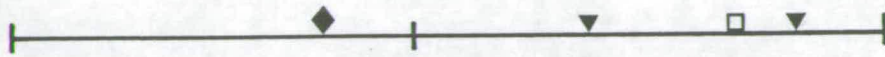
3 Preliminary Analysis of pSP22(1)

(i) Evidence that the Cloned Sequence is Contiguous with the Genomic Sequence

As mentioned in the Introduction it was important to ensure that the cloned fragment was contiguous with the S. pombe genome. To test this, total S. pombe chromosomal DNA was digested with restriction enzymes that cut with the cloned 5.6 Kb fragment. This digested DNA

Figure 4.2 Restriction maps of *cdc22*-M45 complementing plasmids

a. Restriction map of *S.pombe* insert in pSP22(1).

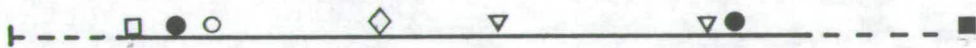


1Kb

No sites for :-

□ BamHIBglI| HindIIIClaI▼ PstIKpnI◆ XbaIPvuIISalIAlso two sites for EcoRI(not mapped).XhoI

b. Restriction map of *S.pombe* insert in pSP22(2).

*S.pombe* DNA

Vector sequence

1Kb

No sites for :-

□ BamHIBglIII◇ BglIClaI▽ EcoRIHpaI| HindIIIPstI○ HincIIPvuII● KpnISstI■ SalIXbaIXhoI

Figure 4.3

Figure 4.3 Restriction Digest Analysis of pDB248 and Plasmid
Obtained from cdc 22 - M45 - GBl(7) (Now Referred to as Plasmid
pSP22(2))

Both pDB248 and pSP22(2) were digested to completion with EcoRI, HindIII and KpnI. The digested plasmids were separated by electrophoresis through a 0.6% agarose gel at 50 V. The gel was then stained and photographed as described in Materials and Methods.

- Lane 1 0.7 μ g of HindIII digested λ DNA, molecular weight markers
- Lane 2 Undigested pSP22(2) (0.5 μ g)
- Lane 3 0.5 μ g HindIII digested pSP22(2)
- Lane 4 0.5 μ g HindIII digested pDB248
- *Lane 5 0.5 μ g EcoRI digested pSP22(2)
- Lane 6 0.5 μ g EcoRI digested pDB248
- Lane 7 0.5 μ g KpnI digested pSP22(2) (partial digest)
- Lane 8 0.5 μ g KpnI digested pDB248
- Lane 9 0.5 μ g pDB248, undigested
- Lane 10 0.7 μ g of HindIII λ DNA, molecular weight markers

* Lane 5 A 0.7 Kb internal EcoRI fragment was too faint to be detected in this gel. However, this fragment can be observed in the autoradiograph shown in Figure 4.6.

was separated on an 0.6% agarose gel, blotted to nitrocellulose and hybridised to radioactively labelled pSP22(1) as detailed in Materials and Methods. If the cloned sequence was contiguous the restriction enzymes should cut the homologous chromosomal DNA at the same sites as on the 5.6 Kb S. pombe fragment.

From the autoradiograph shown in figure 4.4 it can be seen that there were 3 bands homologous to pSP22(1) obtained when total genomic DNA was cut with the restriction enzyme PstI (shown in figure 4.4, track 5). This would correspond to two restriction sites for this enzyme being present in the genomic DNA homologous to the cloned fragment. Similarly, the three homologous bands obtained when total genomic DNA was digested with the restriction enzyme EcoRI (figure 4.4, track 3) would correspond to two sites and the two bands detected when genomic DNA was digested with BamHI (figure 4.4, track 6) would correspond to a single site for this enzyme. The presence of a single band of size 18 Kb with the genomic DNA cut with XhoI indicates that there are no sites for this enzyme in the chromosomal DNA homologous to the cloned fragment. This corresponds to the restriction map of the cloned fragment (See figure 4.2(a)).

In addition if the EcoRI digested pSP22(1) (figure 4.4, track 1) and the EcoRI digested S. pombe genomic DNA (track 3) were compared the internal 2.1 Kb band ran together. This data is consistent with the cloned sequence being contiguous with the S. pombe chromosome.

The autoradiograph also shows that the cdc 22 complementing sequence is present as a single copy in the S. pombe genome as the XhoI digested chromosomal DNA hybridises as a single band to pSP22(1).

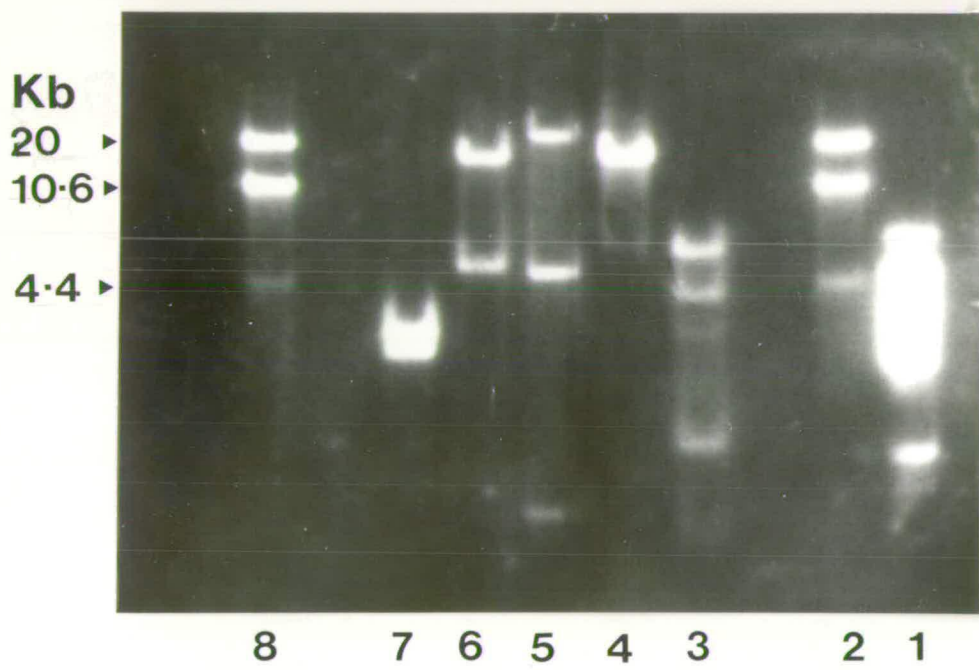


Figure 4.4

Autoradiograph of S. pombe genomic DNA cut with various restriction enzymes separated by electrophoresis on an 0.6% agarose gel, transferred to nitrocellulose and hybridised to radioactively labelled pSP22(1).

- 1 5 ng pSP22(1) cut with EcoRI.
- 2 Molecular weight markers; 20 Kb, 10.6 Kb, 4.4 Kb.
- 3 5 µg genomic DNA cut EcoRI.
- 4 5 µg genomic DNA cut XhoI.
- 5 5 µg genomic DNA cut PstI.
- 6 5 µg genomic DNA cut BamHI.
- 7 5 µg genomic DNA cut HindIII.
- 8 Molecular weight markers.

Lane	Digested with	Major bands	Sizes (Kb)
3	<u>EcoRI</u>	3	* 2.1, 3.9, 5
4	<u>XhoI</u>	1	18
5	<u>PstI</u>	3	* 1.0, 4.7, > 20
6	<u>BamHI</u>	2	4.5, 18
7	<u>HindIII</u>	2	* 2.6, * 3.0

* Small fragment sizes were internal fragments previously estimated by restriction digest analysis of pSP22(1) (see figure 4.1(a)).

(ii) Integration and Mapping

As stated in the Introduction, complementation of a mutation is not sufficient proof that cloned sequences contains the desired gene.

It has been shown from studies with S. cerevisiae that cloned sequences of yeast DNA present on vectors without a yeast origin of replication will only be maintained by integration of the plasmid into the yeast genome. Moreover, this integration occurs predominantly at the region of homology to the yeast DNA sequences. (Struhl et al, 1979, Orr-Weaver et al, 1981). Therefore, stable integration of the cloned fragment into the S. pombe genome followed by genetic mapping to test for linkage to the cdc 22 locus would determine if the cloned sequence contained the cdc 22⁺ gene.

(a) Integration

To determine if pSP22(1) contains the cdc 22⁺ gene, the cloned fragment was subcloned into the integrating vector pESPI. This plasmid contains the S. cerevisiae Leu 2⁺ gene but no origin of replication for S. pombe (See Materials and Methods). The plasmid was integrated into the S. pombe genome and mapped to the cdc 22 locus.

The subcloning was carried out in two different ways:-

- 1 The two HindIII fragments of 2.6 Kb and 3.0 Kb were subcloned separately into pESPI.
- 2 The 5.6 Kb HindIII fragment was subcloned into pESPI.

(b) Subcloning the 2.6 Kb and 3.0 Kb HindIII fragments

The strategy for subcloning the two HindIII fragments is shown in figure 4.5. The plasmids pSP22(1) and pESPI were digested to completion with HindIII. To minimise recircularisation of pESPI twice as much HindIII digested pSP22(1) DNA as HindIII digested pESPI DNA was ligated together. The ligated DNA was used to transform E. coli. Chloramphenicol resistant, tetracycline sensitive colonies were isolated.

Plasmid DNA from these colonies was digested with HindIII and separated by electrophoresis on an 0.6% agarose gel using HindIII digested pSP22(1) as a marker. This showed whether the plasmid contained the 3.0 Kb, 2.6 Kb or both the HindIII fragments. Table 4.1 summarises the results obtained with 130 chloramphenicol resistant E. coli colonies tested.

The plasmids pESPI(3.0) carrying the 3.0 Kb HindIII fragment and pESPI(2.6) carrying the 2.6 Kb HindIII fragment were used to transform a cdc 22-M45 leu 1.32 strain.

A surprising result was that 50 Leu⁺ transformants were obtained per µg of the control pESPI. These transformants were all mitotically unstable and presumably carried the S. cerevisiae Leu 2 gene on an autonomously replicating plasmid. This observation has been investigated by other workers and found to be due to either recombination between plasmids or the acquisition of an S. pombe autonomously replicating sequence from the genome (Wright, A. P. H., 1984).

However, when transforming with pESPI(3.0) and pESPI(2.6) containing an S. pombe DNA insert it was found that the rapidly growing Leu⁺ colonies which grew up first on transformation plates

Figure 4.5

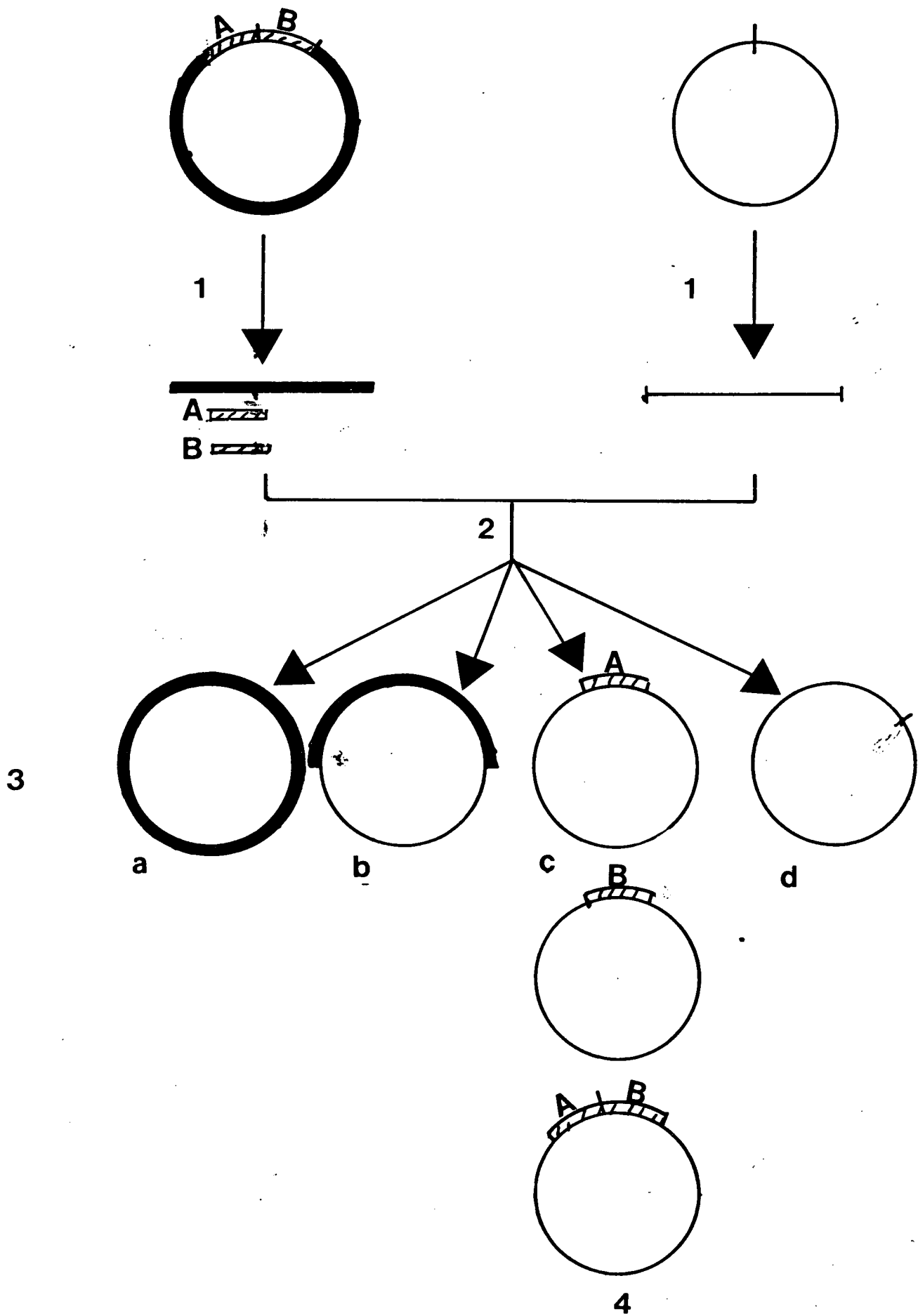


Figure 4.5 Strategy for subcloning the 2.6 Kb and the 3.0 KbHindIII fragments

1. pSP22(1) and pESP1 were digested to completion.
2. The products of digestion (1) were ligated together.
3. The ligated DNA was used to transform E.coli and chloramphenicol resistant, tetracycline sensitive colonies were screened for.
4. Plasmid DNA was prepared from these colonies and subjected to restriction digest analysis to determine which HindIII fragment(s) they contained.

Predicted phenotypes of the different plasmids obtained after step (2)

- a. Chloramphenicol sensitive, tetracycline sensitive.
- b. Chloramphenicol resistant, tetracycline resistant..
- c. Chloramphenicol resistant, tetracycline sensitive.
- d. Chloramphenicol resistant, tetracycline resistant.




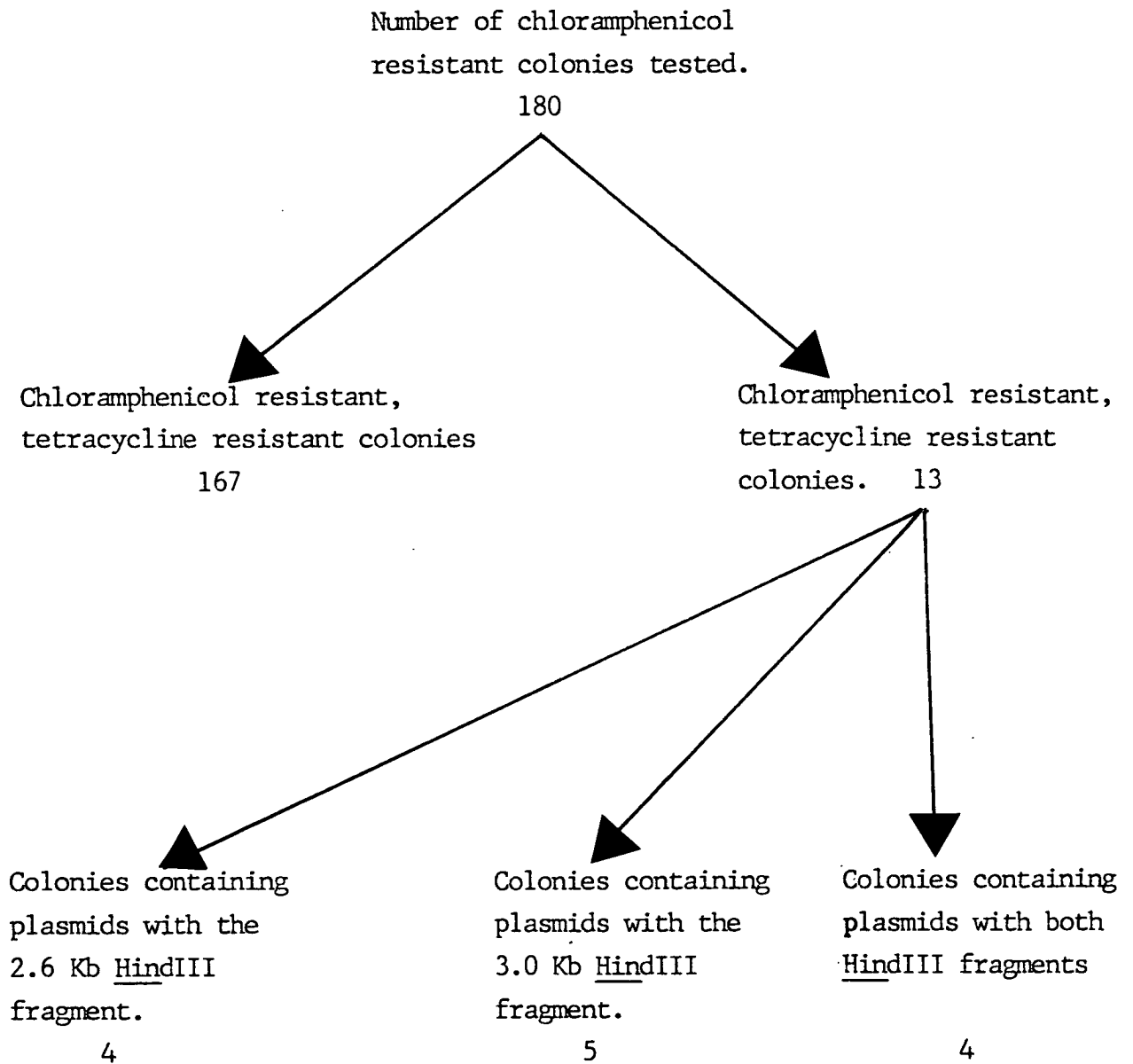
-  pDB262 DNA sequence.
 pESP1 DNA sequence
 S.pombe DNA sequence.

Table 4.1 Characterisation of plasmids obtained from 180 chloramphenicol resistant colonies.



tended to be mitotically stable for leucine prototrophy. This indicated that in these Leu⁺ transformants the plasmid had integrated into the S. pombe genome.

As a rapid test for leucine stability, a single colony was streaked out on a YE plate, and incubated overnight at 25°C. This was then replica plated to YE and grown overnight, three more times in succession. The streak was then replica plated to EMM plus Phloxin and if vigorous growth was obtained along the whole length this was taken to be a presumptive integrant. The integrant was then tested for mitotic stability as detailed in Chapter 3.

Fifteen stable Leu⁺ transformants were obtained. Five were obtained using pESPI(2.6) and ten obtained with pESPI(3.0). None of these transformants complemented the cdc 22-M45 mutation when tested for growth at 35°C.

It has been reported (Caulderon et al, 1983), that certain mutations can be suppressed if extragenic DNA sequences are carried on a high copy number plasmid. This suppression does not occur when the sequence is carried at low copy number either by integration into the chromosome or by its presence in a low copy number plasmid.

It was therefore important to demonstrate that the 3.0 Kb and 2.6 Kb HindIII fragments were unable to complement a cdc 22-M45 mutation when carried on a high copy number plasmid as well as when carried at low copy number integrated in the chromosome. The two HindIII fragments were subcloned into pDB262 by cutting pSP22(1) to completion and religating the digested DNA to itself. This DNA was then used to transform E. coli and tetracycline resistant colonies picked. Plasmid DNA was obtained from these clones, digested with HindIII and the fragments separated on a 0.6% agarose gel using

HindIII digested pSP22(1) as a marker. Plasmids pDB262(2.6.) carrying the 2.6 Kb fragment and pDB262(3.0) carrying the 3.0 Kb HindIII fragment were identified. These plasmids were used to transform a cdc 22-M45 leu 1.32 strain and all the leu⁺ transformants tested were also found to be cdc⁻ when growth was looked for at 35°C. Therefore, the 3.0 Kb and 2.6Kb HindIII fragments are not capable of complementing the cdc 22-M45 mutation at high or low copy number. Therefore, the internal HindIII site of the 5.6 Kb cloned sequence must lie within the DNA sequence required for complementation of the cdc 22-M45 mutation.

I Mapping the site of integration to the cdc 22 locus

Two pESPI(3.0) stable Leu⁺ integrants and two pESPI 2.6 stable Leu⁺ integrants were each crossed to a cdc 22⁺ leu 1.32 strain. The progeny of each cross were analysed by free spore analysis (see Materials and Methods).

The phenotypes of 100 spore colonies were examined for each cross and the results obtained are shown in table 4.2. The Leu⁺ phenotype was used to map the site of integration. The small amount of recombination between the leu⁺ and cdc 22⁺ phenotype as indicated by the low number of cdc⁺ leu⁺ and cdc⁻ leu⁻ colonies obtained shows that the site of integration was strongly linked to the cdc 22 locus.

It is interesting to observe from the data shown in table 4.2 that the amount of recombination obtained with the two integrants transformed with the 2.6 Kb HindIII fragment when crossed to cdc 22⁺ leu 1.32 was greater than with the two integrants transformed with the 3.0 Kb HindIII fragment. Presumably the increased amount of recom-

Table 4.2

Cross	PROGENY TYPES				
	PARENTIAL		RECOMB		% Rec
	<u>cdc</u> ⁺ leu ⁻	<u>cdc</u> ⁻ leu ⁺	<u>cdc</u> ⁺ leu ⁺	<u>cdc</u> ⁻ leu ⁻	
1 pESPI (2.6) - <u>leu</u> ⁺ int (1) x <u>cdc</u> 22 ⁺ leu 1.32	44	47	4	0	4.2
2 pESPI (2.6) - <u>leu</u> ⁺ int (2) x <u>cdc</u> 22 ⁺ leu 1.32	40	45	6	1	7.5
3 pESPI (3.0) - <u>leu</u> ⁺ int (3) x <u>cdc</u> 22 ⁺ <u>leu</u> 1.32	49	48	0	0	0
4 pESPI (3.0) - <u>leu</u> ⁺ int (4) x <u>cdc</u> 22 ⁺ <u>leu</u> 1.32	45	40	1	0	1

ination was observed due to the cdc 22-M45 mutation being present in this HindIII fragment allowing gene conversion and/or recombination to occur.

(c) Subcloning the 5.6 Kb HindIII fragment

To show that the cdc 22 complementing fragment functioned when integrated into the chromosome, the whole 5.6 Kb HindIII fragment was subcloned into pESPI.

Twenty µg of pSP22(1) was partially digested with HindIII and fractionated on a low melting point horizontal bed 1.2% agarose gel. The desired 5.6 Kb fragment was purified by cutting it out of the gel (see Materials and Methods) and ligated into the HindIII site of pESPI. This DNA was used to transform E. coli and colonies that were chloramphenicol resistant and tetracycline sensitive were isolated. Plasmid obtained from these colonies, termed pESPI(56), was used to transform a cdc 22-M45 leu 1.32 strain.

I Integration

Stable integrants were isolated as described before. Five stable Leu⁺ transformants were obtained. When examined at 35°C, four of the transformants complemented the cdc 22-M45 mutation. Presumably, the cdc⁻ leu⁺ transformant arose by gene conversion from the chromosome to the plasmid as it integrated. Gene conversion between a cloned fragment on a plasmid and the homologous DNA on the chromosome has been observed previously in experiments with S. cerevisiae (Falco et al, 1983).

II Mapping the site of integration

A stable cdc⁺ leu⁺ transformant and the cdc⁻ leu⁺ transformant were crossed to a cdc 22⁺ leu 1.32 strain and the asci subjected to tetrad analysis.

In both crosses leucine prototrophy segregated 2 Leu⁺ 2 Leu⁻ showing that the plasmid had integrated into the chromosome.

In the cross involving the cdc⁺ leu⁺ integrant, out of the seventeen tetrads dissected no cdc⁻ colonies were obtained. This shows that the plasmid had integrated within 1.5 centimorgans of the cdc 22 locus. In addition no recombinant tetrads were obtained out of the nineteen asci dissected in the second cross involving the cdc⁻ leu⁺ integrant. This shows that this plasmid had integrated within 1.3 centimorgans of the cdc 22 locus.

The tetrad results confirm that the cloned 5.6 Kb HindIII fragment integrates at the cdc 22 locus and therefore contains the cdc 22⁺ gene of S. pombe.

4 Preliminary Analysis of pSP22(2)

The previous sections demonstrated that the cdc 22 gene was present in the 5.6 Kb S. pombe HindIII fragment contained in pSP22(1). The following sections describe similar experiments used to characterise the 2.2 Kb S. pombe fragment contained in pSP22(2).

(i) Evidence that the Cloned Sequence is Contiguous with the *S. pombe* Genome

Wild type *S. pombe* chromosomal DNA was digested separately with the restriction enzymes EcoRI, and HindIII. The digested DNA was separated on an 0.6% agarose gel, blotted to nitrocellulose and hybridised to radioactivity labelled pSP22(2) as detailed in Materials and Methods.

From the autoradiograph shown in Figure 4.6, it can be seen that there were three bands homologous to pSP22(2) when total genomic DNA was cut with the restriction enzyme EcoRI (Figure 4.6, track 3). This corresponds to two restriction enzyme sites being present for this enzyme on the genomic DNA homologous to the cloned fragment. The presence of a single band with HindIII indicates that there are no restriction sites for this enzyme and that the gene is present as a single copy in the genome (Figure 4.6, track 4). In addition the internal 0.7 Kb EcoRI band was present in the genomic DNA and pSP22(2) cut with EcoRI (Figure 4.6, tracks 2 and 3).

These results demonstrate that the cloned fragment is colinear in the chromosome and that no part of the sequence was due to a ligation artifact formed during the construction of the gene library.

(ii) Integration and Mapping

(a) Subcloning into pESPI

The procedure for subcloning the 2.2 Kb complementing fragment is shown in Figure 4.7. Both pESPI and pSP22(2) were digested to

Figure 4.6

Autoradiograph of genomic S. pombe DNA cut with EcoRI and HindIII, separated by electrophoresis on a 0.6% agarose gel, transferred to nitrocellulose and hybridised to radioactively labelled pSP22(2).

Lane

- 1 Molecular weight markers of size 10.6 Kb, 6.3 Kb, 4.3 Kb.
- 2 pSP22(2) cut with EcoRI (5 ng).
- 3 5 µg of genomic DNA digested with EcoRI.
- 4 5 µg of genomic DNA digested with HindIII.

Figure 4.7

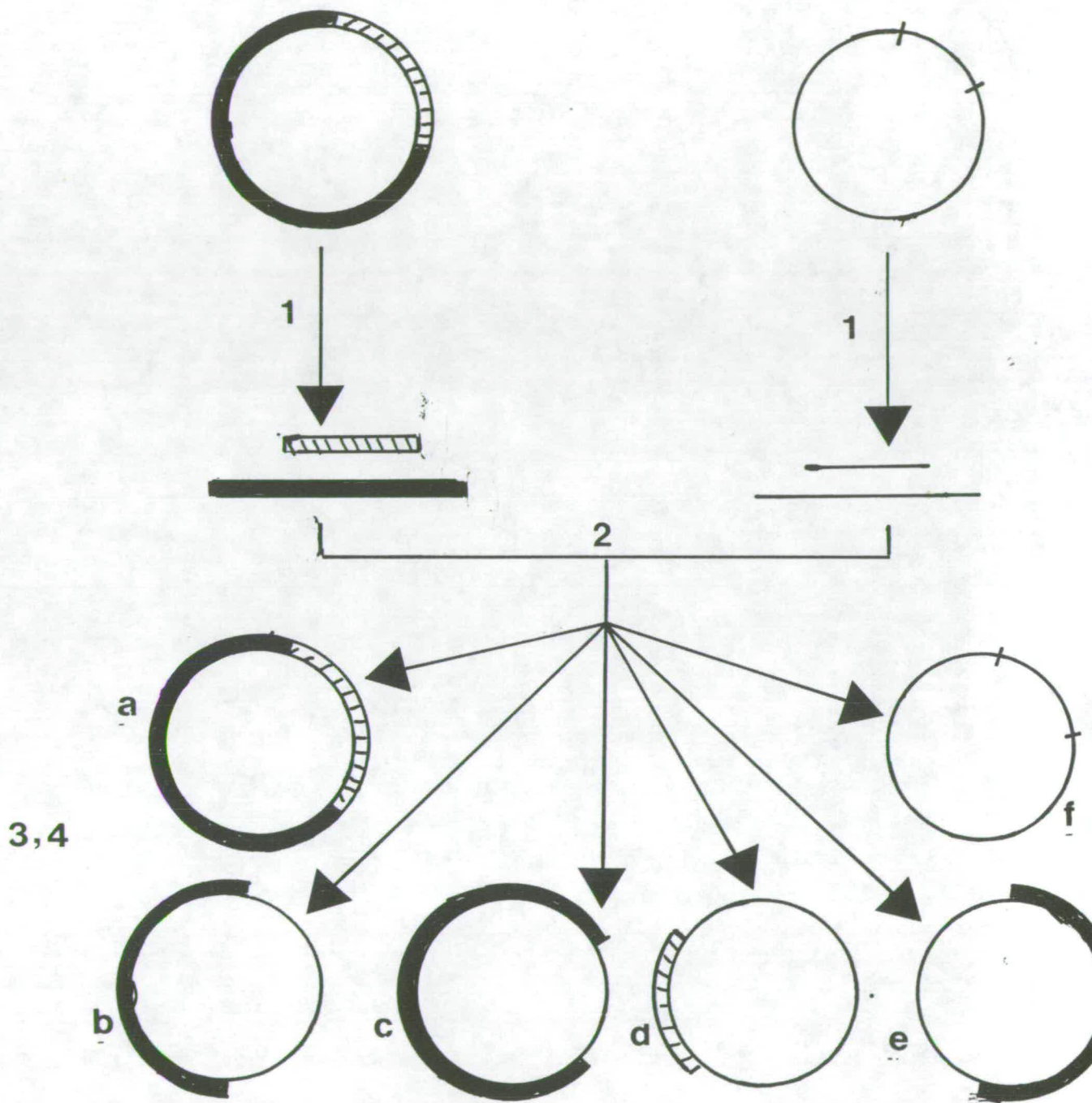





Figure 4.7 Strategy for subcloning the 2.2Kb fragment from pSP22(2)
into pESPl

1. pSP22(2) and pESPl are cut with SalI and HindIII to completion.
2. Digested pSP22(2) and pESPl are ligated together.
3. The ligated DNA was used to transform E.coli.
4. Chloramphenicol resistant, tetracycline sensitive colonies were screened for.

Predicted phenotypes of the different plasmids obtained after step (2).

- a. Chloramphenicol resistant, tetracycline sensitive, ampicillin resistant.
- b. Chloramphenicol sensitive, tetracycline sensitive, ampicillin resistant.
- c. Chloramphenicol sensitive, tetracycline sensitive, ampicillin resistant.
- d. Chloramphenicol resistant, tetracycline sensitive, ampicillin sensitive.
- e. Chloramphenicol resistant, tetracycline sensitive, ampicillin resistant.
- f. Chloramphenicol resistant, tetracycline resistant, ampicillin sensitive.

-  pDB248 DNA sequence
 pESPl DNA sequence
 S.pombe DNA sequence

completion with the restriction enzymes SalI and HindIII. Equal amounts of both digested DNA were ligated together. This DNA was used to transform E. coli. By selecting for chloramphenicol resistance, tetracycline sensitivity and ampicillin sensitivity only colonies containing the desired plasmid termed pESPI(2.2)A were obtained.

(b) Integration

Plasmid pESPI(2.2)A was used to transform a cdc 22-M45 leu 1.32 strain. From 100 Leu⁺ transformants tested, nine stable integrants were obtained. All nine were cdc⁻ when checked for growth at 35°C.

(c) Mapping

Four stable cdc⁻ leu⁺ integrants were crossed to a cdc 22⁺ leu 1.32 strain. The data obtained from tetrad analysis is shown in table 4.3. The presence of recombinant tetrads, that is non-parental ditype (NPD) and tetratype (TT) asci, in the progeny of each cross indicates that the site of integration and the cdc 22 locus are not closely linked.

Although no homology to the leu 1 gene can be detected under high stringency by Southern blots using the leu 2 gene of S. cerevisiae (see figures 4.4 and 4.6), integration might nevertheless have occurred at this locus. To test this linkage of the leu⁺ phenotype to the mating type locus was calculated. The data shown in table 4.4 shows that the introduced Leu⁺ marker is unlinked to the mating-type locus. The leu 1 locus is 13 centimorgans from the mating type locus (Kohli et al, 1977) therefore if integration had occurred at this site some linkage

Cross	No. Asci	Ascus Type			Leu ⁺ :Leu ⁻	% Rec
		PD	NPD	T		
Int - pSP22(2) - 1 x <u>cdc 22⁺ leu 1.32</u>	13	5	1	7	2:2	34.6
Int - pSP22(2) - 2 x <u>cdc 22⁺ leu 1.32</u>	7	2	2	3	2:2	50
Int - pSP22(2) - 3 x <u>cdc 22⁺ leu 1.32</u>	17	4	2	11	2:2	44
Int - pSP22(2) - 4 x <u>cdc 22⁺ leu 1.32</u>	5	1	2	2	2:2	60

Parental Ditype (PD) cdc⁻ leu⁺; cdc⁺ leu⁻

Non-parental ditype (NPD): cdc⁺ leu⁺; cdc⁻ leu⁻

Tetratype (TT) : cdc⁻ leu⁺; cdc⁺ leu⁻; cdc⁺ leu⁺; cdc⁻ leu⁻

Table 4.4 Linkage of Ints to Mating type Locus

Cross	No. Asci	Ascus Type			Leu ⁺ :Leu ⁻	% Rec
		PD	NPD	T		
Int - pSP22(2) - 1h ⁻ x <u>leu 1.32 h⁺</u>	13	2	4	7	2:2	57
Int - pSP22(2) - 2h ⁻ x <u>leu 1.32 h⁺</u>	7	-	2	5	2:2	64
Int - pSP22(2) - 3h ⁻ x <u>leu 1.32 h⁺</u>	15	1	1	13	2:2	50

Tetrad Type

Phenotypes

Parental ditype (PD) : leu⁺ h⁻; leu⁻ h⁺

Non-parental ditype (NPD): leu⁺ h⁺; leu⁻ h⁻

Tetra type (TT) : leu⁺ h⁻; leu⁻ h⁺; leu⁺ h⁺; leu⁻ h⁻

would have been observed.

The genetic data given above show that the site of integration is unlinked to the leu 1 and cdc 22 loci. Therefore, provided that the plasmid has integrated by homology, the cloned fragment cannot contain the cdc 22 gene.

To demonstrate that the cloned DNA had integrated by homology, chromosomal DNA was prepared from a cdc 22-M45 leu 1.32 strain and from two stable integrants. The DNA was digested with BamHI which cuts once at the border between the vector and the cloned fragment (see figure 4.3). After Southern transfer, the digested DNA was probed with radioactively labelled pSP22(2). From the autoradiograph shown in figure 4.8 the single BamHI fragment of about 20 Kb in size homologous to pSP22(2) was converted by the integration of pESPI(2.2)A to two bands. One of these was 5 Kb in size: the other was greater than 20 Kb. The fragments produced by integration can be explained using the model shown in figure 4.9. The 2.2 Kb cloned S. pombe fragment (shown as a thick line) is contained within the 20 Kb BamHI chromosomal fragment. When pESPI(2.2)A of total length 12.3 Kb integrates by homologous recombination a tandem duplication of the 2.2 Kb fragment is formed with the vector sequences of pESPI located in between. As pESPI(2.2)A contains only one BamHI site situated at the border between the vector and the cloned fragment, two new BamHI fragments are generated with a combined length of 32.3 Kb made up of the original BamHI chromosomal fragment of 20 Kb and pESPI(2.2)A of 12.3 Kb. The sizes of the two BamHI fragments generated can be explained if it is assumed that the 2.2 Kb fragment is located 2.8 Kb and 15 Kb from the chromosomal BamHI sites. These results demonstrate that the pESPI(2.2)A

Figure 4.8

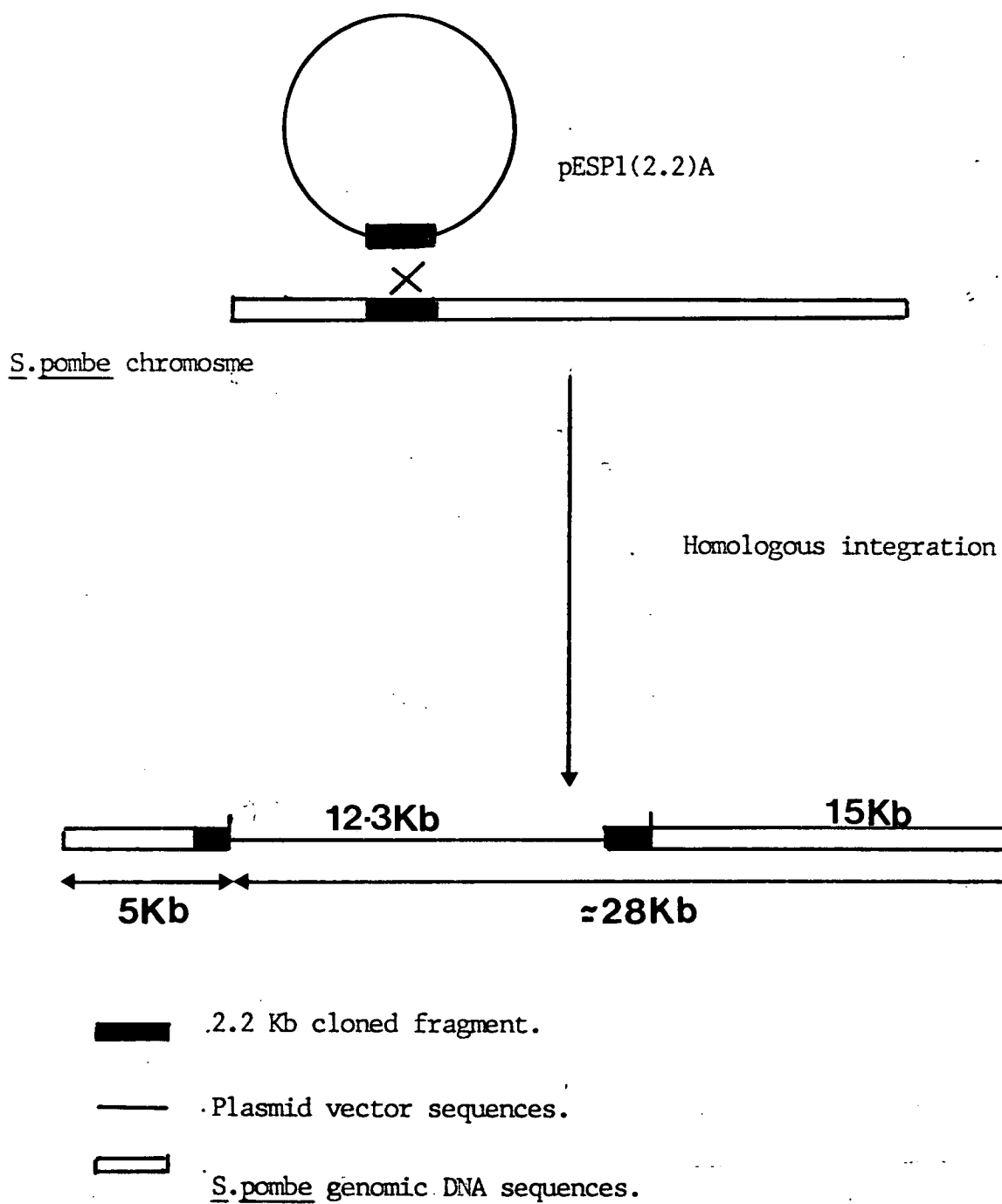


Autoradiograph of genomic DNA cut with BamHI, separated on a 0.6% agarose gel, transferred to nitrocellulose and hybridised to radioactively labelled pSP22(2).

- 1 5 μ g of genomic DNA from a cdc 22-M45 leu 1.32 cut with BamHI.
- 2 5 μ g of genomic DNA from a cdc 22-M45 leu 1.32 strain transformed by integration with pESPI(2.2)A cut with BamHI.
- 3 Same as (2).

Δ start of gel

Figure 4.9 - Model of integration of pESP1(2.2)A



had integrated by homology. In addition, the autoradiograph also demonstrates that only one copy of the plasmid had integrated. This is because multiple integration would generate a third band the size of the pESP1(2.2)A plasmid, 12.3 Kb in size, which is not observed (Figure 4.8).

Therefore the cloned fragment present in pSP22(2) contains a suppressor of cdc 22-M45 which complements only when carried on the high copy number vector pDB248. The plasmid pSP22(2) is now designated pSup22.

5 Lack of Complementation of Other cdc Mutants with pSup22

The previous section demonstrated that pSup22 does not carry the cdc 22 but an extragenic suppressor of this gene. Therefore, it was possible that pSup22 carried the gene for one of the other cdc mutants that had been isolated in S. pombe. To test this possibility, leu 1.32 was crossed into a number of G₁ and S phase S. pombe cdc mutants, transformed with pSup22, and the Leu⁺ transformants obtained were tested for growth at 35°C on EMM. From the results shown in Table 4.5 for the 15 cdc mutations tested only the two cdc 22 alleles were suppressed by pSup22 as demonstrated by allowing growth at 35°C.

Table 4.5

STRAIN	CELL CYCLE BLOCK	COMPLEMENTATION WITH pSup22	REFERENCE
<u>cdc</u> 1.7 <u>leu</u> 1.32	Mitosis	-	Nurse <u>et al</u> , 1976
<u>cdc</u> 2.33 <u>leu</u> 1.32	G ₁ /Mitosis	-	"
<u>cdc</u> 10.129 <u>leu</u> 1.32	G ₁	-	"
<u>cdc</u> 20 M10 <u>leu</u> 1.32	S phase	-	Nasmyth <u>et al</u> , 1981
<u>cdc</u> 21 M68 <u>leu</u> 1.32	S phase	-	"
<u>cdc</u> 22 M45 <u>leu</u> 1.32	S phase	+	"
<u>cdc</u> 22 C11 <u>leu</u> 1.32	S phase	+	See Chapter 7
<u>cdc</u> 23 M36 <u>leu</u> 1.32	S phase	-	Nasmyth <u>et al</u> , 1981
<u>cdc</u> 24 <u>leu</u> 1.32	S phase	-	"
* <u>cdc</u> -C1 <u>leu</u> 1.32	-	-	
<u>cdc</u> -C2 <u>leu</u> 1.32	-	-	
<u>cdc</u> -C4 <u>leu</u> 1.32	-	-	
<u>cdc</u> -C41 <u>leu</u> 1.32	-	-	
<u>cdc</u> -C43 <u>leu</u> 1.32	-	-	
<u>cdc</u> -C47 <u>leu</u> 1.32	-	-	

Key: + growth at 35°C
 - no growth at 35°C

* Mutations isolated independently during the course of this work by Dr Jim Creanor. The transition points of each of these mutants was before or at S phase.

The six plasmids able to complement cdc 22 mutations contained two different S. pombe DNA sequences.

The plasmids obtained from cdc 22-GB2(1) to cdc 22-GB2(4) all contain an identical 5.6 Kb insert. This sequence was contiguous with the genomic sequence and present as a single copy in the S. pombe genome. When integrated, the 5.6 Kb fragment complemented the cdc 22-M45 mutation and mapped to the cdc 22 locus. Therefore, the cdc 22 gene was present in this sequence.

The plasmids obtained from cdc 22-GB1(7) and cdc 22-GB1(10) both contained the same 2.2 Kb insert. The sequence was contiguous and present as a single copy in the S. pombe chromosome. When integrated, it did not complement the cdc 22-M45 mutation or map to the cdc 22 locus. Southern blot analysis showed that the cloned fragment had integrated by homology and it was concluded that it contained a suppressor of the cdc 22-M45 mutation.

The suppressor does not complement most other known S phase or G₁ S. pombe mutations.

CHAPTER 5 Transcriptional Analysis of the cdc 22 Gene

1 Introduction

Using the cloned gene as a hybridisation probe to detect homologous RNA transcripts, a number of parameters were determined for the cdc 22 gene.

Transcript size was obtained by comparing the mobility of the homologous RNA to that of known size markers.

The transcriptional orientation of the cdc 22 gene was identified by finding which region of the cloned DNA contained the cdc 22 promoter.

The cell cycle dependency of transcription of the cdc 22 gene could be determined using cdc mutants.

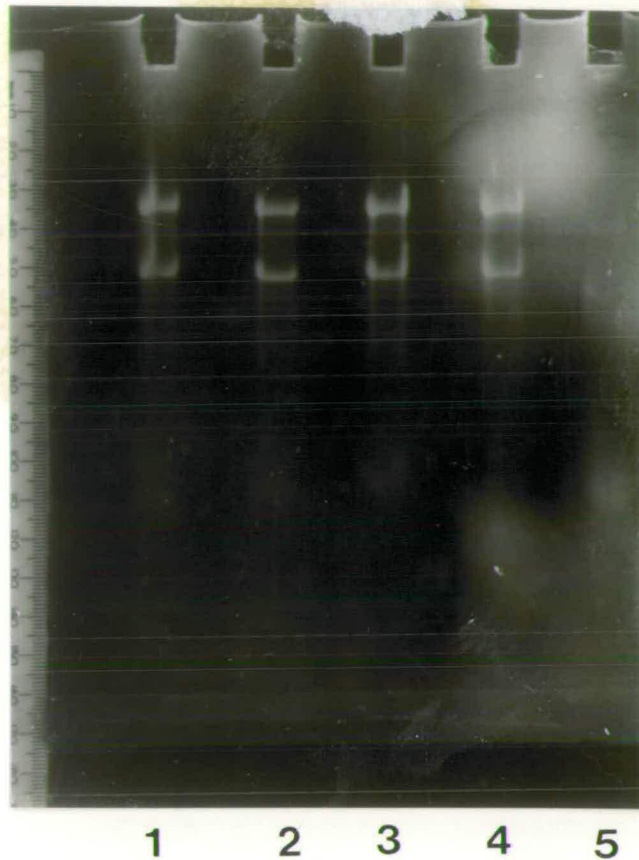
Finally, the level of the cdc 22 transcript through the cell cycle was estimated from RNA obtained from a synchronous culture.

2 Detection of the cdc 22 Transcript

The cloned 5.6 Kb DNA fragment present in pSP22 that complements cdc 22 mutation was used as a hybridisation probe to detect the transcript encoded by the cdc 22⁺ gene.

Total RNA was prepared from a 972h⁻ wild type strain grown in YEPD at 25°C overnight by mechanically breaking the cells with glass beads in the presence of phenol as detailed in Materials and Methods. Forty µg of total S. pombe RNA and 5 µg of E. coli total RNA were fractionated by electrophoresis on a 1.2% formaldehyde denaturing agarose gel. A ruler was placed alongside the gel to determine the mobility of the S. pombe and E. coli rRNA bands which were used as size markers. A photograph of the gel is shown in Figure 5.1. A strip containing separated total RNA from wild type S. pombe was

Figure 5.1 Total RNA from *S.pombe* and *E.coli*



40 μ g of total *S.pombe* RNA isolated from a wild type (972) strain and 5 μ g of total *E.coli* RNA were separated by electrophoresis on a 1.2% denaturing agarose gel. The molecular ^{size} ~~weights~~ (Kb) of each ribosomal RNA band are shown. The positions in the gel of the rRNA bands were detected by staining with ethidium bromide.

Lanes 1 to 4: 40 μ g of *S.pombe* RNA

* Lane 5: 5 μ g of *E.coli* RNA

*The *E.coli* rRNA bands were only faintly visible in the original photograph. Their positions relative to the *S.pombe* rRNA's are shown in figure 5.1. ▲

cut from the gel using a scalpel. The RNA was transferred to nitrocellulose and the blot hybridised to radioactively labelled pSP22 (See Materials and Methods). The autoradiograph obtained is shown in Figure 5.2 and detects only one homologous transcript to the cloned S. pombe DNA sequence. By plotting the known molecular weight markers on a graph of log (molecular weight) against mobility (shown in Figure 5.3), a molecular weight of 3.3 Kb was estimated for the homologous transcript.

It has already been shown in Chapter 4 that the internal HindIII restriction enzyme site in the 5.6 Kb fragment (see Figure 4.1) lies within the cdc 22 gene. This was deduced from the fact that the two subclones of the 5.6 Kb fragment generated by using the internal HindIII site of 3.0 Kb and 2.6 Kb in size were not able to complement the cdc 22 mutation. The cdc 22 transcript would be expected to overlap this internal HindIII site and be detected by both the 3.0 Kb and 2.6 Kb HindIII subcloned fragments when used as a hybridisation probe. Therefore, if the 3.3 Kb transcript detected using the 5.6 Kb fragment as a hybridisation probe was coded by the cdc 22 gene this transcript would be detected when both the 3.0 Kb and 2.6 Kb fragments were used as hybridisation probes.

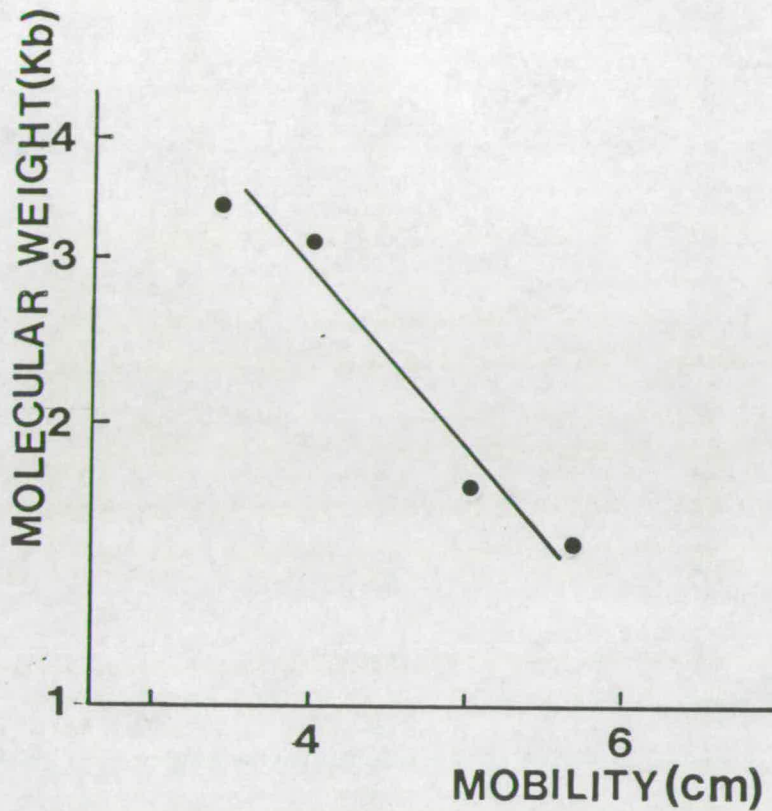
To test this, total RNA from wild type cells was separated on a denaturing gel and subjected to Northern blot analysis using either pDB262(3.0) containing the 3.0 Kb HindIII fragment, pDB262(2.6) containing the 2.6 Kb HindIII fragment, pSP22 or pDB262 as hybridisation probes. The autoradiograph shown in Figure 5.4 shows that no transcript was homologous to pDB262 (Figure 5.4, Lane 4), but that a single transcript of 3.3 Kb in size was homologous to pDB262 (3.0), pDB262(2.6) and pSP22 (Figure 5.4, Lanes 1, 2, 3). Thus this result gives conclusive

Figure 5.2 Detection of Transcript Homologous to pSP22

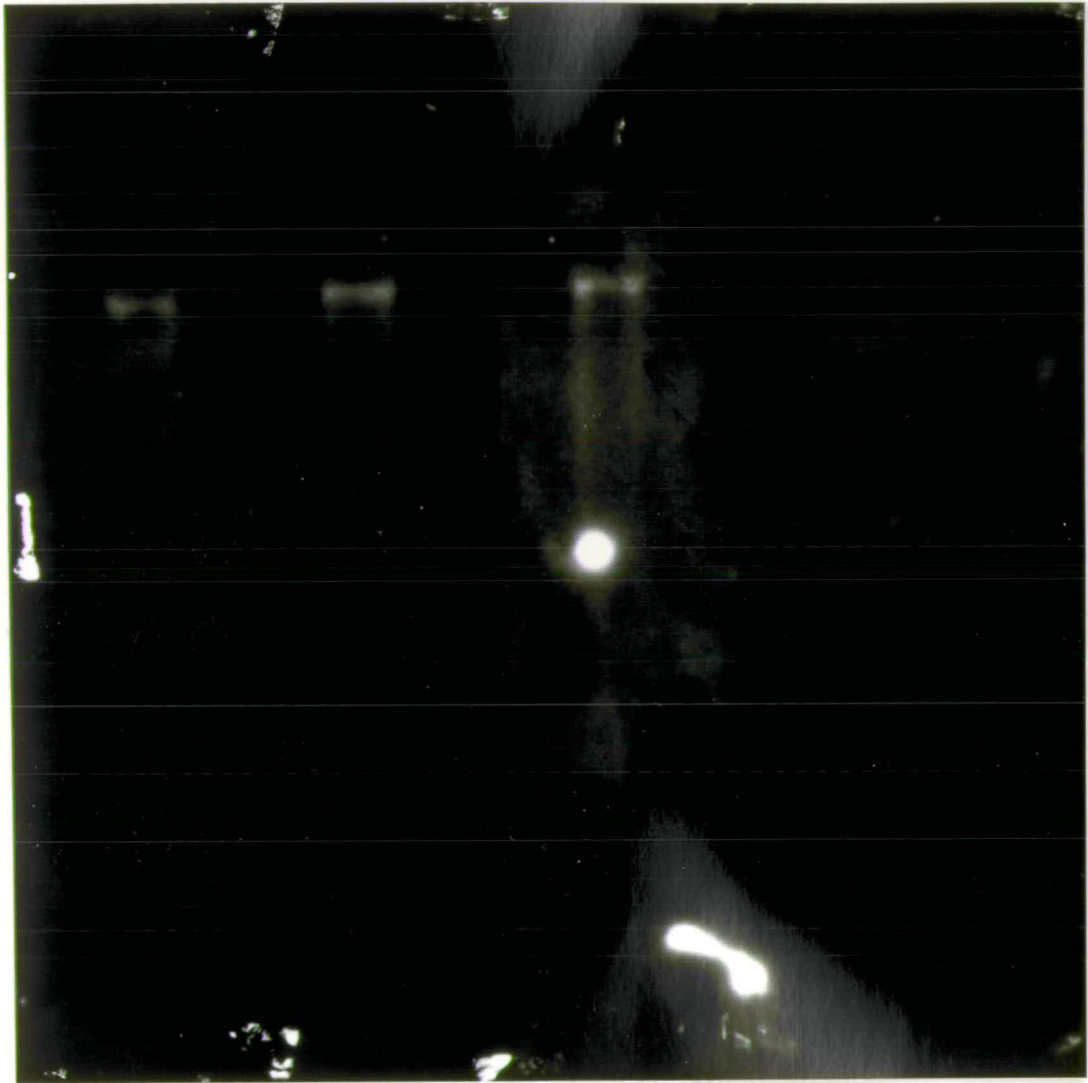


40 μ g of total *S. pombe* RNA was separated on a denaturing gel, transferred to nitrocellulose and hybridised to radioactively labelled pSP22.

Figure 5.3 Determination of the molecular weight of the *cdc22* mRNA.



Using the data shown in figure 5.1, the log of the molecular weight of the *S.pombe* ribosomal RNA (1.7Kb and 3.4Kb; Philippsen *et al*, 1978) and the *E.coli* ribosomal RNA (1.5Kb and 3.2Kb; Birmacombe *et al*, 1978) were plotted against their mobilities (cm) through the agarose gel. Using this graph the size of the *cdc22* mRNA was estimated from the distance the *cdc22* transcript travelled through the gel.



1

2

3

4

Figure 5.4 Hybridisation of pSP22, pDB262(2.6); pDB262(3.0) and pDB262 to *S. pombe* RNA

40 µg of total RNA isolated from a wild type (972) strain was separated on a 1.2% denaturing agarose gel, transferred to nitrocellulose and hybridised to the DNA probes shown. The position of the rRNA molecular weight markers (in Kb) were detected prior to transfer by staining with ethidium bromide.

Lane 1: *S. pombe* RNA hybridised to pDB262(2.6)

Lane 2: *S. pombe* RNA hybridised to pDB262(3.0)

Lane 3: *S. pombe* RNA hybridised to pSP22.

Lane 4: *S. pombe* RNA hybridised to pDB262

proof that the 3.3 Kb transcript was transcribed by the cdc 22 gene. In addition, it can be concluded that the internal HindIII must lie within the transcribed region of the cdc 22 mRNA and not in the 5' or 3' flanking regions.

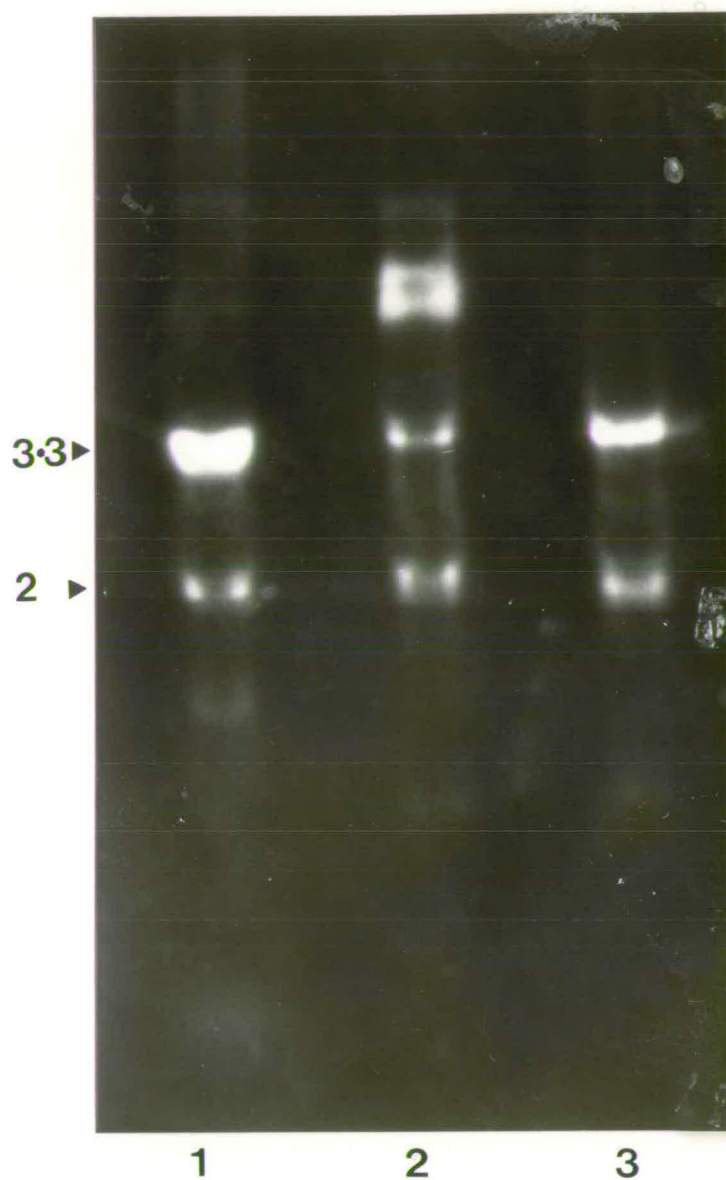
3 Direction of Transcription of the cdc 22 Gene

To determine the transcriptional orientation of the cdc 22 gene, the two plasmids pDB262(3.0) and pDB262(2.6) that each contain part of the cdc 22 gene were assayed for the presence of the cdc 22 S. pombe promoter. The fragment containing the promoter of the cdc 22 gene would contain the 5' end allowing the transcriptional orientation to be deduced.

To assay for the presence of the cdc 22 promoter the two plasmids were used to transform independently a cdc 22 leu 1.32 strain. Total RNA was made from Leu⁺ transformants carrying the plasmids. This RNA was separated by electrophoresis on a denaturing gel and subjected to Northern blot analysis using pSP22 as a hybridisation probe. For comparison RNA from an integrant transformed with pESP1(5.6) was also used in the experiment.

From the autoradiograph obtained (Figure 5.5) it can be seen that two homologous transcripts of 2 Kb and 3.3 Kb in size were detected in all three RNA preparations.

The 3.3 Kb transcript was coded for by the chromosomal cdc 22 gene. It was present in greatest abundance in the RNA preparation obtained from the integrant strain and probably reflects the increased copy number of the cdc 22 gene in this strain. (figure, 5.5; Lane 1). For some unknown reason this transcript was present at a reduced level

Figure 5.5

Lane	Transformed with	Major bands	Sizes (Kb)
1	pESP1(5.6)	2	2.0,3.3
2	pDB262(3.0)	4	2.0,3.3,6.0,7.2
3	pDB262(2.6)	2	2.0,3.3,

Figure 5.5 Direction of Transcription of the cdc 22 Gene

Total RNA was isolated from a cdc 22 - M45 leu 1.32 strain transformed with pDB262(2.6); pDB262(3.0) or pESP1(5.6) (integrating vector). 40 µg of RNA from each transformant strain was separated on a 1.2% denaturing agarose gel, transferred to nitrocellulose and hybridised to radioactively labelled pSP22. Molecular weights of the RNA transcripts detected were determined by comparison with ribosomal RNA's.

Lane 1: RNA isolated from cdc 22 - M45 leu 1.32 transformed with pESP1(5.6)

Lane 2: RNA isolated from cdc 22 - M45 leu 1.32 transformed with pDB262(3.0⁴)

Lane 3: RNA isolated from cdc 22 - M45 leu 1.32 transformed with pDB262(2.6)

in the strain transformed with pDB262(3.0) compared to that transformed with pDB262(2.6) (Figure 5.5; Lanes 2 and 3).

The 2 Kb transcript was probably coded for by the S. cerevisiae Leu 2⁺ gene which was the selectable marker present in all three vectors and used to select for S. pombe transformants. It was surprising to find that this transcript was present in the same abundance in all three transformed strains. This was because two of the plasmids used, pDB262(3.0) and pDB262(2.6), from studies in S. cerevisiae would be expected to be present in a high copy number of 25 to 100 copies per cell (Gerbaud et al., 1979 ; Chevallier et al., 1980), compared to the integrant strain where one extra copy had integrated, as had been determined previously by Southern blot analysis (see Figure 4.7). However this ambiguity can be explained by the recent work of Wright et al who have shown that vectors based on the S. cerevisiae 2 μ m plasmid are maintained at levels of less than 5 copies per cell in S. pombe (Dr A Wright, Personal Communication).

Two other transcripts of 7.2 Kb and 6 Kb in size were only detected in the RNA preparation obtained from the pDB262(3.0) transformed strain. This data would be consistent with the cdc 22 S. pombe promoter being present on this 3.0 Kb cloned fragment. The large transcript sizes could be explained by the absence of the cdc 22 transcription termination site in the subclone pDB262(3.0). This would allow transcription to carry on into the plasmid coding sequences until a termination site was reached. Presumably, two transcripts were obtained due to different termination of transcription signals being used in the vector coding sequences. An alternative explanation of the two transcripts may be that two promoters were present in the 3.0 Kb fragment that directed

transcription in opposite directions to each other. One would correspond to the cdc 22 promoter while the other would direct transcription of an unknown gene located next to the cdc 22 gene in the S. pombe chromosome. The different sized transcripts could be explained by allowing transcription of each transcript to continue into the plasmid coding region until a transcription termination site was reached.

In summary, the 3.3 Kb transcript detected was coded by the cdc 22 gene located on the S. pombe chromosome. The 2 Kb transcript was coded by the S. cerevisiae Leu 2⁺ gene present on the vector sequences of each plasmid. In addition, the transcription of the 6 Kb and 7.2 Kb transcript was initiated within the 3.0 Kb cloned S. pombe insert present in pDB262(3.0). No transcription was initiated from the cloned S. pombe 2.6 Kb fragment found in pDB262(2.6).

Thus the autoradiograph shown in Figure 5.5 is consistent with the 5' end and promoter of the cdc 22 gene being located within the 3.0 Kb HindIII fragment while the 3' end and transcription termination sequences are found in the 2.6 Kb HindIII fragment. The exact positions of these transcriptional control regions cannot be deduced from the above data.

4 Cell Cycle Dependency of the Appearance of the cdc 22 Transcript

If cells are blocked in the nuclear division cycle either by a genetic mutation or a chemical inhibitor, subsequent cell cycle events that are dependent on the completion of the blocked stage do not occur. Thus cells that are blocked before or during DNA synthesis do not undergo mitosis and vice versa (Mitchison, 1971). Growth, that is, the accumulation of protein, RNA and other macromolecules

however continues relatively unaffected.

The cdc 22 gene product is involved in the initiation of DNA synthesis. It was therefore possible that the appearance of the cdc 22 transcript was, like DNA synthesis, dependent on the completion of a specific cell cycle event(s).

To test this possibility cdc mutants that arrested at various points in the cell cycle were blocked by incubating them at their restrictive temperature of 36°C for 4½ hours. The mutant strains used, together with the stage in the cell cycle at which they blocked at the restrictive temperature are shown in Table 5.1. Samples were removed for microscopic observation to ensure that the cells had the characteristic S. pombe cdc phenotype of elongated cells without cell plates.

RNA was made from each blocked mutant and 40 µg separated by electrophoresis on a 1.2% denaturing agarose gel. The RNA was then transferred to nitrocellulose and hybridised to radioactively labelled pSP22.

The autoradiograph shown in Figure 5.6 shows that the 3.3 Kb mRNA corresponding to the cdc 22 transcript was detected in the RNA prepared from each blocked mutant strain. The mRNA was detected in approximately equal abundance in the total RNA obtained from each mutant strain. (In the RNA obtained from the blocked cdc 20 strain there appears to be half as much of the 3.3 Kb transcript present, however this was due to 20 µg of RNA being loaded instead of 40 µg).

The autoradiograph therefore shows that the appearance of the cdc 22 transcript is not dependent on the completion of a specific cell cycle event. The significance of this is discussed in relation to cell cycle control(s) in Chapter 9.

Table 5.1

Mutant Strains used and where they block in the Cell Cycle for Dependency Experiments

MUTANT STRAIN	STAGE IN CELL CYCLE BLOCKED AT 36°C	REF
<u>cdc</u> 1:2	Mitosis	Nurse <u>et al</u> , 1976
<u>cdc</u> 2.33	G ₁ and Mitosis	"
<u>cdc</u> 10-129	G ₁	"
<u>cdc</u> 17-M75	S phase	"
<u>cdc</u> 20-M10	Initiation of S phase	Nasmyth <u>et al</u> , 1981
<u>cdc</u> 22-M45	" " " "	"

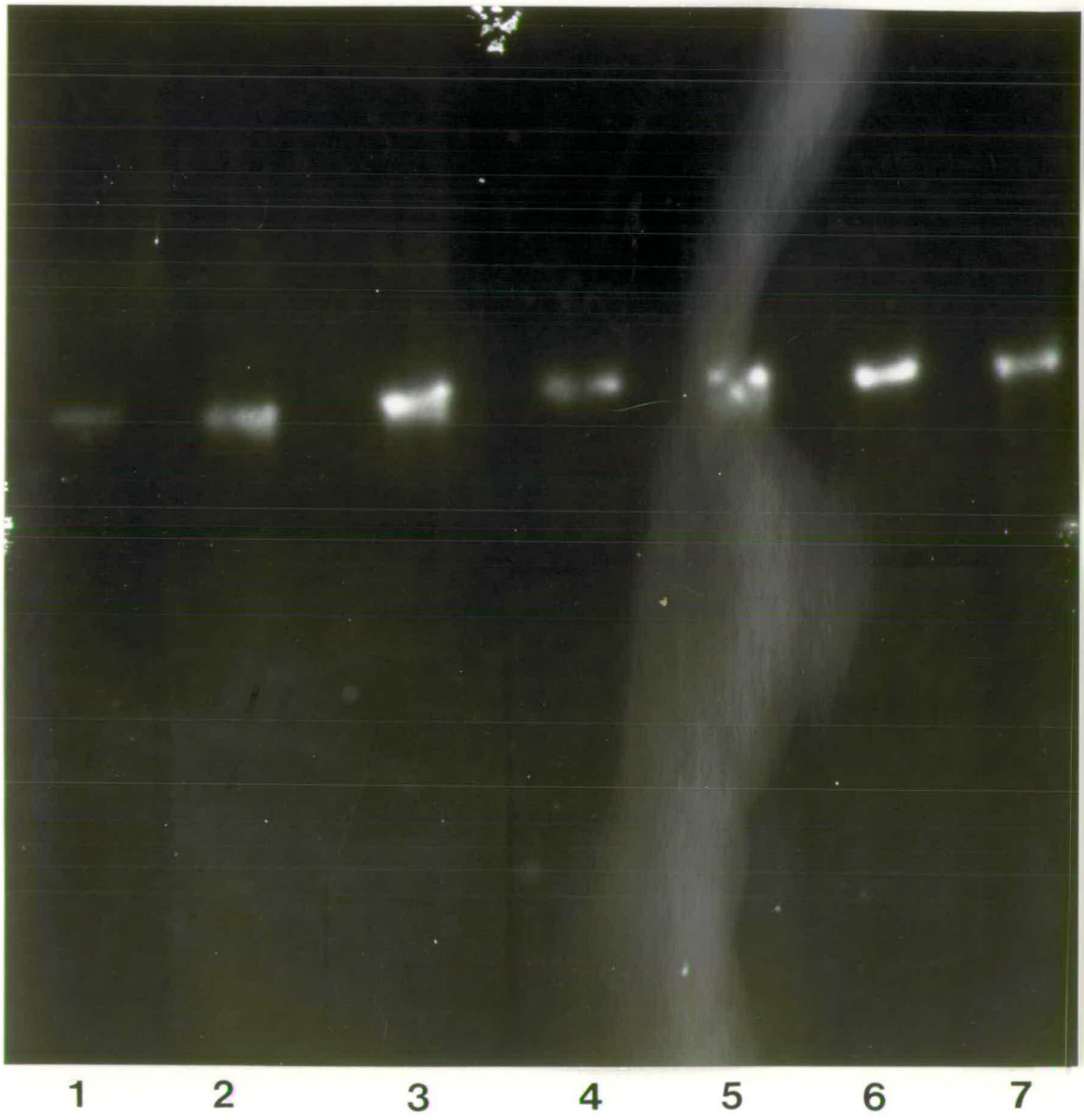


Figure 5.6 Cell Cycle Dependency of the Accumulation of the *cdc 22* Transcript

The strains shown below were shifted from the permissive to the restrictive temperature and incubated for 4½ hours (approximately two cell cycles), after which RNA was isolated as detailed in Materials and Methods. Total RNA from each strain was separated by electrophoresis on a 1.2% denaturing agarose gel, transferred to nitrocellulose and hybridised to radioactively labelled pSP22.

Lane

- 1 20 µg of RNA from *cdc 20* - M10
- 2 40 µg of RNA from *cdc 22* - M45
- 3 40 µg of RNA from *cdc 17* - M75
- 4 40 µg of RNA from *cdc 1.7*
- 5 40 µg of RNA from *cdc 2.33*
- 6 40 µg of RNA from *cdc 10.129*
- 7 40 µg of RNA from wild type (972)

5 Level of cdc 22 Transcript during the Cell Cycle

In order to investigate the level of the cdc 22 mRNA through the cell cycle RNA samples were prepared from wild type cells growing as a synchronous culture. The synchronous culture was obtained by size selection on an elutriating rotor (See Materials and Methods). This method of achieving synchrony is reported to reduce the perturbations inherent in most other synchronisation procedures (Creanor and Mitchison, 1979; discussed in detail in the Introduction). Small cells were selected and allowed to grow at 35°C for two generations as measured by the increase in cell number. The cell number curve also gave an estimate of the degree of synchrony. RNA samples were prepared from approximately 2×10^7 cells as detailed in Materials and Methods.

The cell number curve of the synchronous culture used is shown in Figure 5.7. Two stepwise increases in cell number are observed indicating a high degree of synchrony. Samples for the preparation of RNA were taken every 15 minutes as detailed in Materials and Methods. 5 µg of total RNA was separated by gel electrophoresis and blotted to gene screen nylon membrane. The RNA blot was hybridised to radioactively labelled pSP22 DNA, which contained the cdc 22 gene. The autoradiograph obtained is shown in Figure 5.8. The autoradiograph clearly demonstrates that the cdc 22 transcript exhibited dramatic changes in its level during the cell cycle. The cdc 22 transcript is originally present at a low level rising to a peak by the fourth sample, before returning to a low level by sample 8. The amount of cdc 22 transcript remains at a low level until sample 12 is reached when the cdc 22 mRNA once more rises to a maximum level by sample 14, returning to a

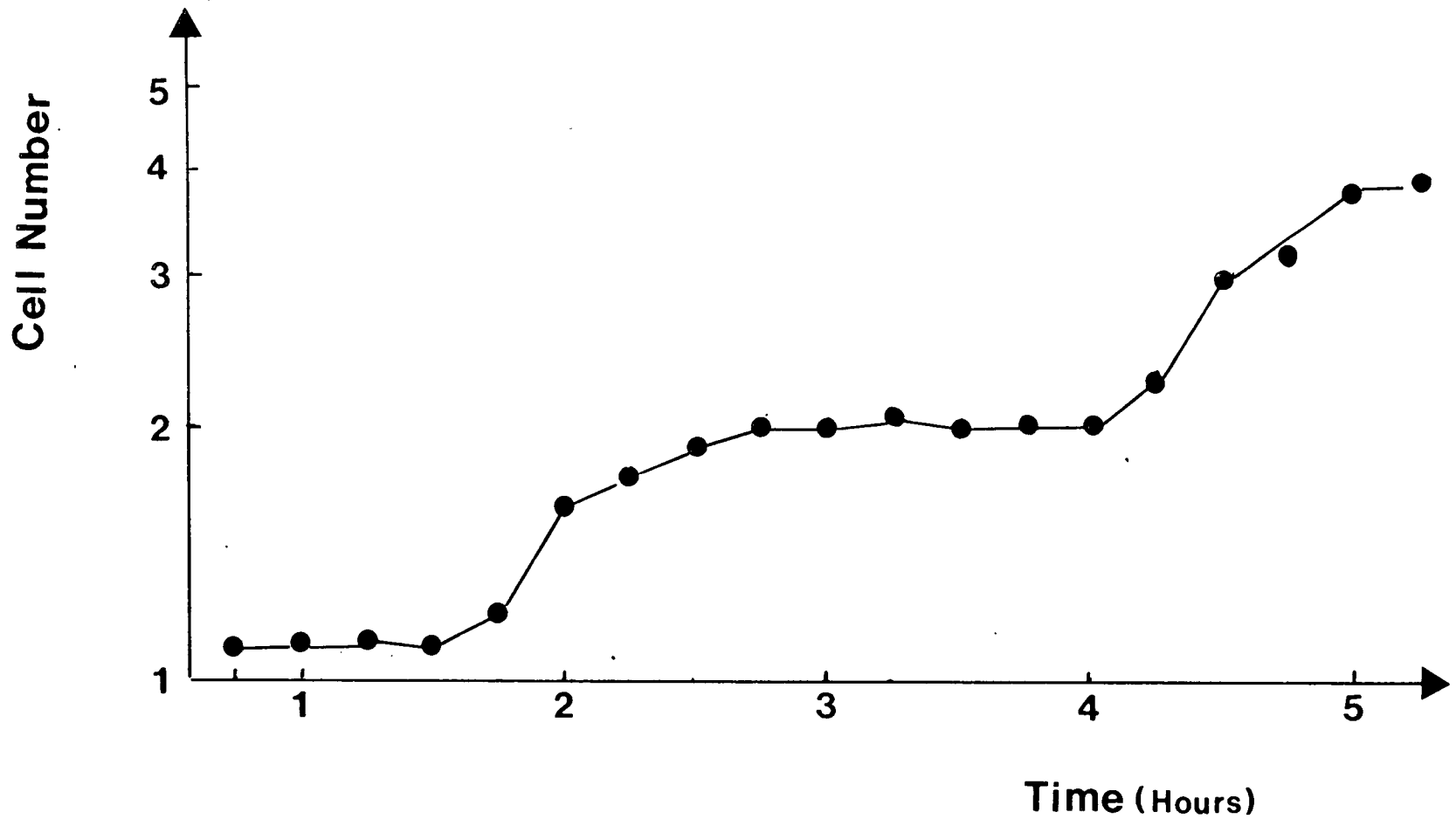
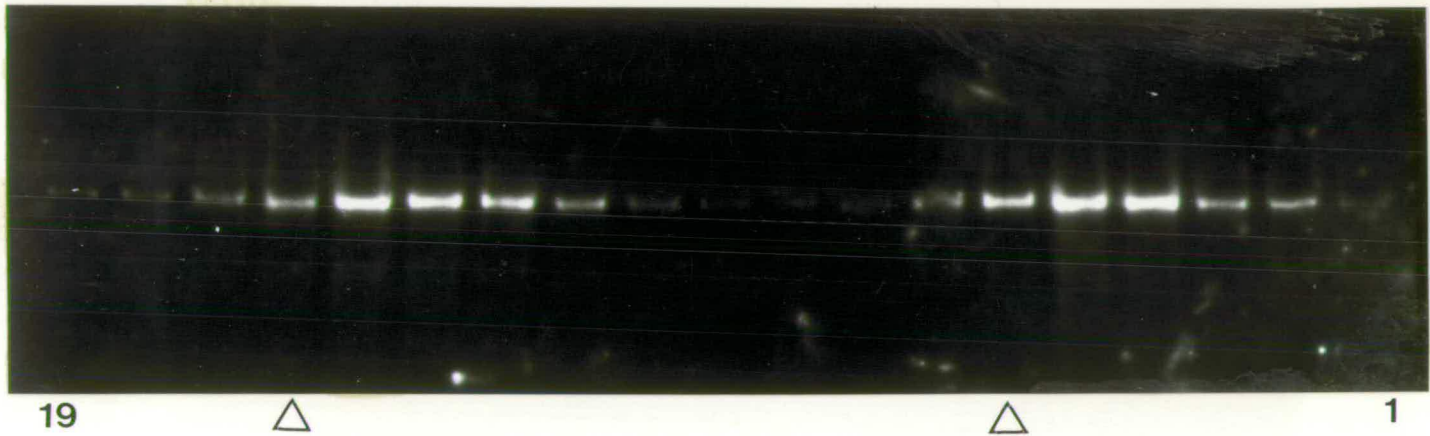


Figure 5.7 Cell Number of Wild Type Synchronous Culture Incubated at 35°C

The synchronous culture was sampled every 15 minutes, 45 minutes after selection from the elutriating rotor. Cell number was determined from a 0.1 ml sample as described in Materials and Methods. The cell number is given in arbitrary log units. One unit equals 1×10^6 cells ml^{-1} .

Figure 5.8 Cell cycle regulation of the level of the cdc22 ^{transcript.} ~~transport.~~



△ mid point of cell number increase (from Figure 5.7)

a cdc 22 mRNA

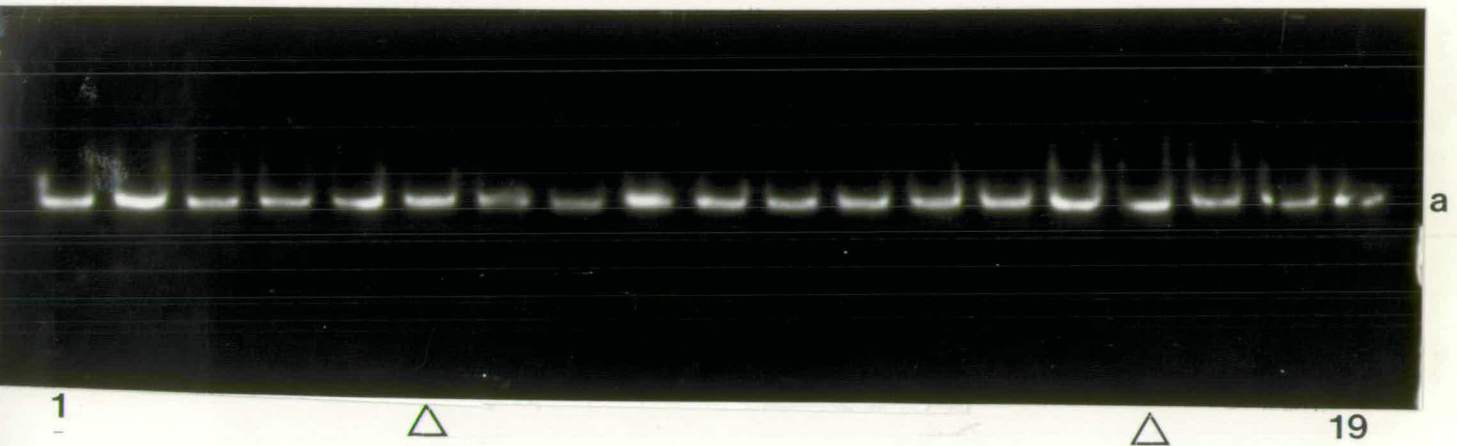
Lane 1 First RNA preparation obtained from cells sampled 45 minutes after selection from the elutriator rotor.

Samples were removed for RNA preparation every 15 minutes from a synchronous culture of wild type S. pombe cells growing at 35°C. 5 µg of each sample was separated by electrophoresis on a 1.2% denaturing agarose gel, transferred to gene screen and hybridised to radioactively labelled pSP22.

basal level for the duration of the experiment. Although difficult to measure, the difference in abundance between the low and high levels of each transcript was estimated by eye to be at least tenfold.

The time between the appearance of the two peaks is approximately $2\frac{1}{2}$ hours which corresponds to the generation time of wild type cells at 35°C , the temperature at which the synchronous culture was incubated. This strongly suggests that the level of the cdc 22 transcript is cell cycle regulated. The time in the cell cycle at which the level of the cdc 22 transcript peaks can be estimated from the cell number curve (shown in Figure 5.7). The peaks are observed approximately 30 minutes before the midpoint of the cell number rise. This is equivalent to the cells being in late G_1 or early S phase of the cell cycle at this point.

The alcohol dehydrogenase transcript has been previously reported to be present at the same level during the cell cycle (Aves et al, 1984). Therefore as an aperiodic control the same filter was reprobed with the radioactively labelled plasmid pADH (obtained from Dr Paul Nurse) containing the S. pombe alcohol dehydrogenase gene. The autoradiograph obtained is shown in Figure 5.9 and demonstrates that the level of the alcohol dehydrogenase transcript unlike the cdc 22 transcript, does not vary dramatically during the cell cycle.

Figure 5.9 Level of the ADH mRNA through the Cell Cycle

Same blot as shown in Figure 5.8 reprobed with pADH as a periodic control.

△ mid point of cell number rise

a ADH mRNA

Lane 1 First RNA preparation obtained from cells sampled 45 minutes after selection from the elutriator rotor.

6 Summary

The 5.6 Kb HindIII DNA fragment which contains the cdc 22 gene is homologous to a single mRNA transcript of 3.3 Kb in size.

The 3.0 Kb HindIII subclone contains the 5' end of the cdc 22 gene, and the 2.6 Kb HindIII subclone the 3' end.

The appearance of the cdc 22 transcript is not dependent on the completion of a specific cell cycle event.

The level of the cdc 22 transcript is cell cycle regulated, reaching a maximum value during late G₁ to early S phase of the cell cycle.

CHAPTER 6 Transcription Analysis of the cdc 22 Suppressor

1 Introduction

Essentially, the same experiments were carried out as described in the previous chapter using pSup22 as a hybridisation probe to determine the transcript size, and cell cycle dependency of the cdc 22 suppressor sequence. The plasmid pSup22 contains the 2.2 Kb S. pombe DNA sequence that suppresses a cdc 22 - M45 mutation cloned in the vector pDB248.

2 Transcript size

To determine the size of the cdc 22 suppressor transcript Northern blot analysis of wild type total RNA was carried out using pSup22 as a hybridisation probe.

Total RNA was prepared from a 972h⁻ (wild type) strain grown at 25°C in YEPD as described previously. Forty µg samples of S. pombe and 5 µg of E. coli total RNA were separated by electrophoresis on a 1.2% denaturing agarose gel. A photograph was taken of the rRNA as before and the RNA on the gel subjected to Northern analysis using pSup22 as a hybridisation probe. The autoradiograph obtained is shown in Figure 6.1. From this autoradiograph only one transcript homologous to pSup22 could be detected. Its size was estimated to be approximately 1.5 Kb by comparison of its mobility with that of the E. coli and S. pombe rRNAs (See Figure 6.2).

3 Cell Cycle Dependency of the Appearance of the cdc 22 Suppressor Transcript

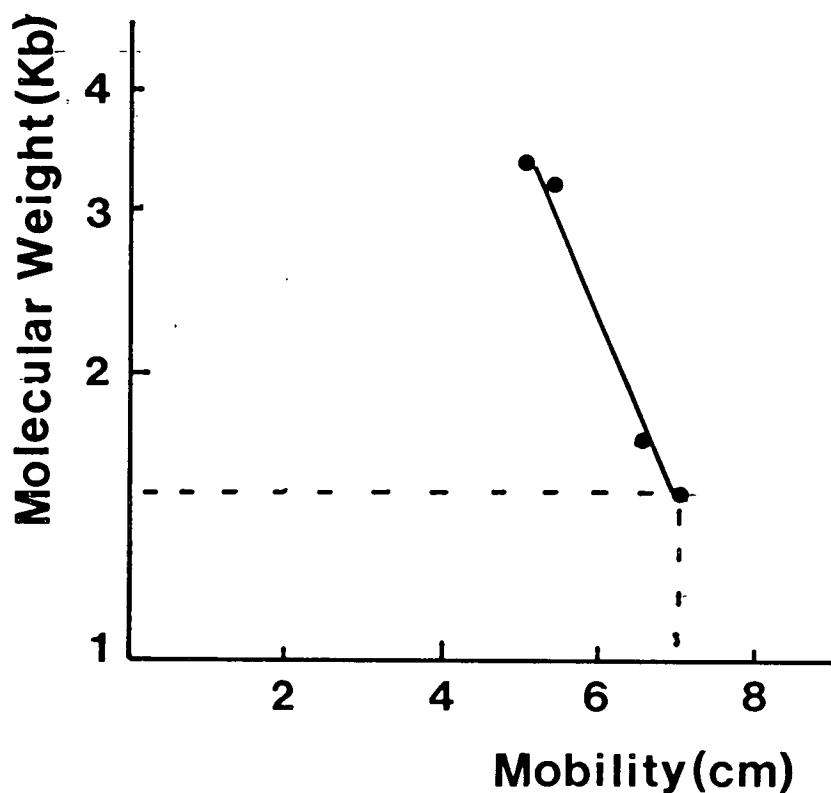
The rationale behind this experiment was identical to that

Figure 6.1 Hybridisation of pSup22 to S.pombe RNA



40 μ g of total RNA isolated from wild type (972) S.pombe was separated on a 1.2% denaturing agarose gel, transferred to nitrocellulose and hybridised to pSup22 DNA. The position of the E. coli and S. pombe rRNA were used as molecular weight markers (Kb).

Figure 6.2



The E.coli and S.pombe rRNA bands were used as molecular weight markers to estimate the size of transcripts homologous to pSup22. The method used was identical to that described in figure 5.3 to estimate the size of the CDC22 transcript.

discussed in the last chapter to determine the cell cycle dependency of the accumulation of the transcript. Total RNA was prepared from cells blocked at different points in the cell cycle by using cdc mutant strains (See Table 5.1). Forty μ g of RNA from each blocked mutant along with forty μ g from wild type cells grown at the permissive temperature of 25°C were separated by electrophoresis on a denaturing gel and subjected to Northern blot analysis using pSup22 as a probe.

From the autoradiograph shown in Figure 6.3 it can be observed that the 1.5 Kb homologous transcript was detected at similar levels in the RNA from each strain. However, in the case of the RNA prepared from blocked cdc 1.7, cdc 10.129, cdc 17 - M75, cdc 20 - M10, and cdc 22 - M45 mutant strains an additional homologous transcript of approximately 2.4 Kb in size was detected. This transcript was present at approximately the same level in each of these strains. By comparing the two transcripts by eye the 2.4 Kb transcript was estimated to be half as abundant as the 1.5 Kb transcript.

Surprisingly, the larger 2.4 Kb transcript was not detected in the RNA prepared from the blocked cdc 2.33 strain and from wild type cells grown at 25°C. A possible explanation for these unexpected results could be that the extra transcript was obtained when cells are incubated at the higher temperature, that is, the appearance of the larger transcript was due to a heat shock response. Production of heat shock transcripts has been well documented in a number of organisms including the budding yeast S. cerevisiae (Ashburner and Bonner, 1979; Ellwood and Craig, 1984). This could be easily tested by looking for the presence of a 2.4 Kb transcript in RNA made from wild type cells growing at the restrictive temperature of 36°C. Alternatively, the difference could be explained by the appearance of

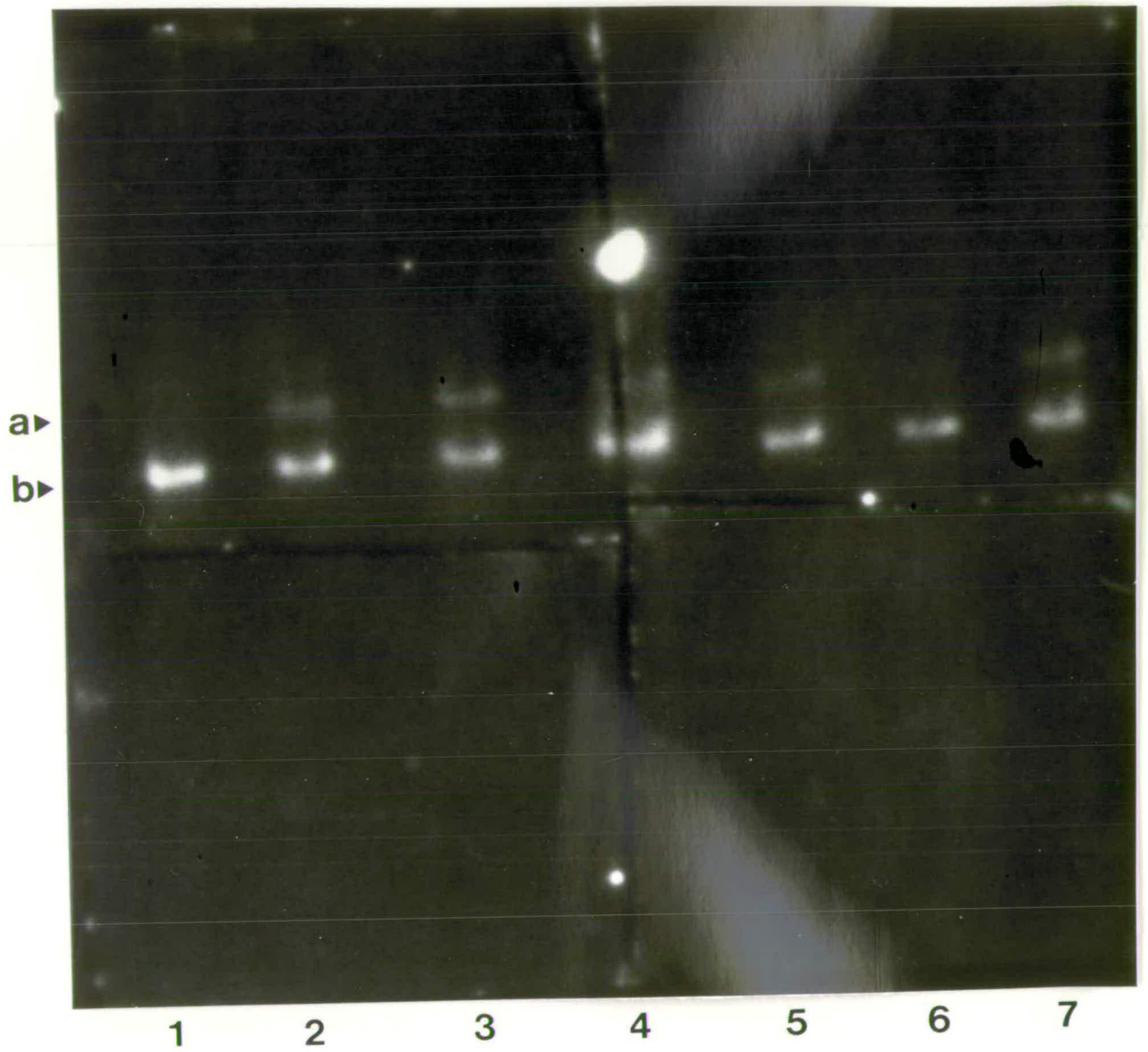


Figure 6.3 Cell Cycle Dependency of the Appearance of Transcripts Homologous to pSup22

40 µg of total RNA isolated from various blocked cdc mutant strains and wild type cells grown at 25°C were separated by gel electrophoresis. The RNA was transferred to nitrocellulose and hybridised to radioactively labelled pSup22. Position of S. pombe and E. coli rRNA's used as molecular weight markers.

- Lane 1: S. pombe wild type RNA
Lane 2: S. pombe cdc 10.129 RNA
Lane 3: S. pombe cdc 20-1110 RNA
Lane 4: S. pombe cdc 22 - M45 RNA
Lane 5: S. pombe cdc 17 - M75 RNA
Lane 6: S. pombe cdc 2.33 RNA
Lane 7: S. pombe cdc 1.7 RNA

a 2.4 Kb transcript

b 1.5 Kb transcript

the 2.4 Kb transcript being a response to cell cycle arrest. However, with both these interpretations the larger transcript would be expected to be present at the same level in a blocked cdc 2.33 strain compared to the other blocked cdc mutants. The absence of an increased amount of the larger 2.4 Kb transcript in RNA obtained from a cdc 2.33 blocked mutant suggests that the cdc 2 gene product maybe required for the appearance of the larger transcript.

To investigate these possibilities the following experiment was carried out. A 100 ml YEPD flask containing either a cdc 2.33, cdc 1.7 or 972 wild type strain was grown overnight at 25°C to an optical density of 0.2 as read at a wavelength of 595 nm. The cdc 1.7 strain was taken to be typical of the five cdc mutants in which the 2.4 Kb transcript could be detected when incubated at 36°C. Each culture was then split in half, 50 mls shifted to 36°C and the remaining 50 mls left at 25°C. After 4½ hours the cells were harvested and RNA was prepared from each culture as before. 40 µg of each RNA preparation was then separated by electrophoresis and subjected to Northern blot analysis using pSup22 as a probe.

The autoradiograph obtained is shown in Figure 6.4. This shows that the 1.5 Kb homologous transcript is present at the same level in each RNA preparation irrespective of temperature.

However, the additional transcript of 2.4 Kb in size was detected only in the RNA prepared from the cdc 1.7 strain that had been incubated at 36°C. As before, this transcript was present at approximately half the abundance of the 1.5 Kb transcript. In the other RNA preparations the 2.4 Kb transcript was only detected after prolonged exposure of the blot and was estimated to be present at less than a tenth of the level in the RNA obtained from the cdc 1.7 strain incub-

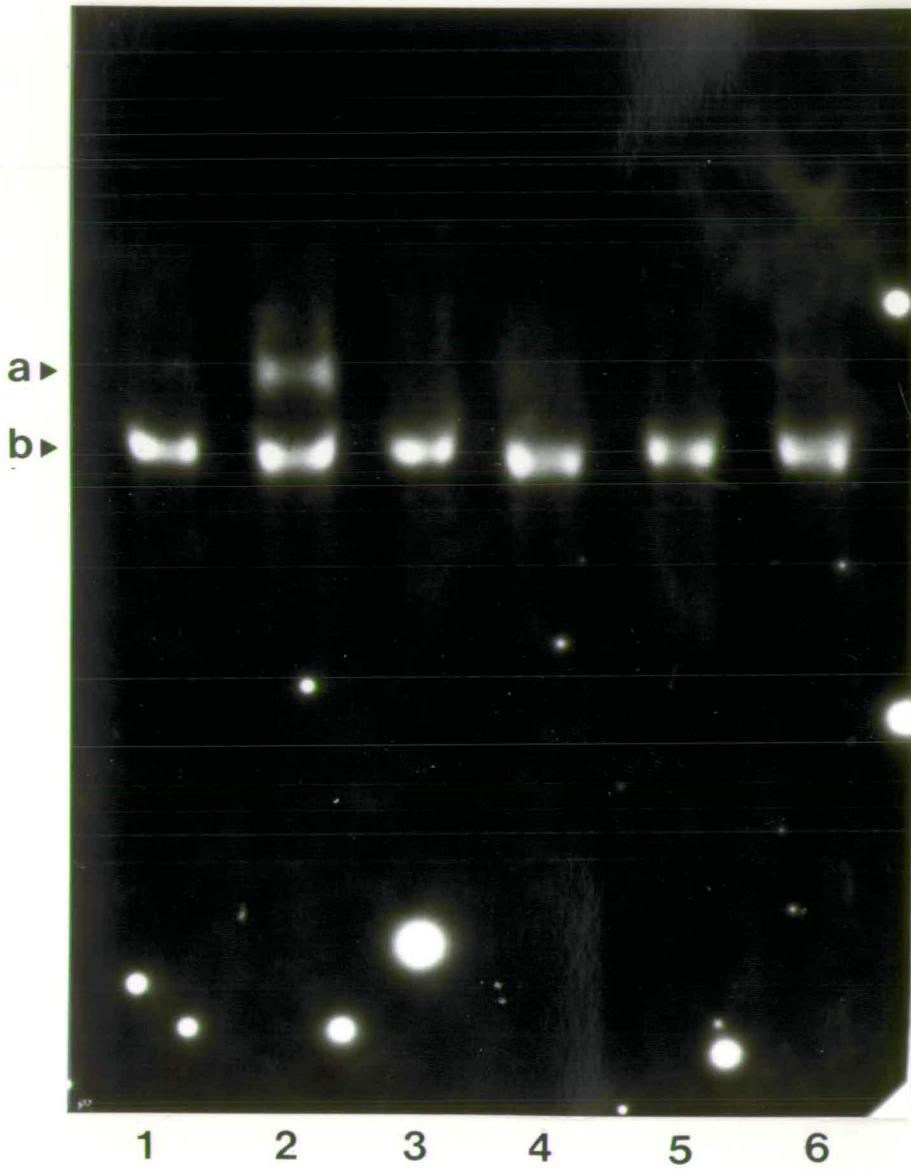
Figure 6.4

Figure 6.4

As detailed in the text 100 mls YEPD containing either a cdc 2.33, cdc 1.7 or 972 strains was grown overnight at the permissive temperature (25°C). Each culture was then split in half, 50 mls shifted up to the restrictive temperature (36°C) and 50 mls left at the permissive temperature. After 4½ hours (approximately equal to two cell cycles at the restrictive temperature) RNA was prepared from each culture as described in Materials and Methods. 40 µg of each RNA sample was separated by electrophoresis through a 1.2% alkaline denaturing agarose gel, transferred to nitrocellulose and hybridised to radioactively labelled pSup22. The autoradiograph obtained is shown.

Lane

- 1 40 µg of RNA prepared from cdc 1.7 incubated at 25°C.
- 2 40 µg of RNA prepared from cdc 1.7 incubated at 36°C.
- 3 40 µg of RNA prepared from cdc 2.33 incubated at 25°C.
- 4 40 µg of RNA prepared from cdc 2.33 incubated at 36°C.
- 5 40 µg of RNA prepared from 972 (wild type) incubated at 25°C.
- 6 40 µg of RNA prepared from 972 (wild type) incubated at 36°C.

a 2.4 Kb transcript

b 1.5 Kb transcript

ated at 36°C.

Therefore, it was concluded that the 2.4 Kb transcript was not produced as a response to the heat shock of incubation at 36°C as the transcript was not present at an elevated level in RNA prepared from wild type cells incubated at 36°C. The autoradiograph also suggests that the appearance of the large transcript in the RNA prepared from blocked cdc 1.7, cdc 10.129, cdc 17 - M75, cdc 20 - M10, and cdc 22 - M45 strains was a result of blocking the cell cycle in these strains. This is because the larger transcript could only be detected at a elevated level when cell cycle arrest occurred in these strains. The absence of the 2.4 Kb transcript in a blocked cdc 2.33 strain is an intriguing observation, especially as the cdc 2 gene product has been shown to play an important role in cell cycle controls during G₁ and G₂ (Nurse and Thuriaux, 1980; Nurse and Bisset, 1981). Possible models to account for the appearance of the 2.4 Kb transcript in the various strains tested are considered in the discussion.

4 Level of the Transcripts Homologous to pSup 22 through the Cell Cycle

To investigate the possibility that either the 1.5 Kb or the 2.4 Kb transcripts were cell cycle regulated a blot of RNA obtained from a synchronous culture of wild type cells was hybridised to radioactively labelled pSup22. The autoradiograph obtained is shown in Figure 6.5. From this autoradiograph, only the 1.5 Kb transcript can be easily detected. The 2.4 Kb transcript was present at a low abundance in each sample and was not visualised in Figure 6.5. In contrast to the cdc 22 mRNA no dramatic fluctuations in the level of the 1.5 Kb transcript was observed.

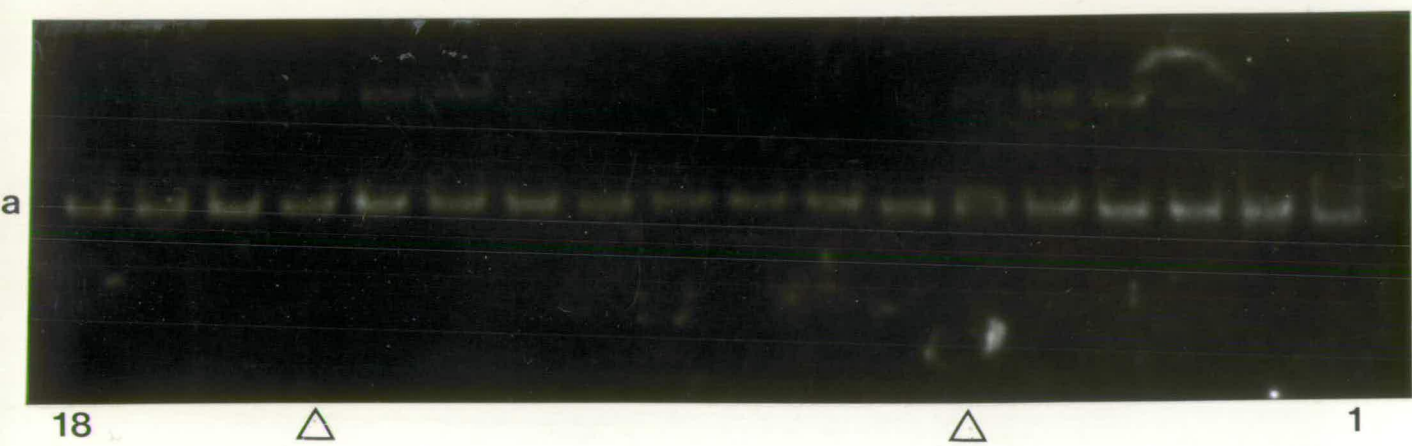


Figure 6.5 Level of transcripts homologous to pSup22 through the cell cycle.

The same RNA blot as described in figure 5.8 was rehybridised to radioactively labelled pSup22

△ denotes the midpoint of the cell number rise

a 1.5 Kb transcript

Lane 1 First RNA preparation obtained from cells sampled 40 minutes after selection from elutriator rotor

5 Summary

The 2.2 Kb DNA fragment which contains the cdc 22 suppressor gene is homologous to two transcripts, of 1.5 Kb and 2.4 Kb in size.

The 1.5 Kb transcript was detected in six cdc mutant strains and a 972 wild type strain at both the permissive and the restrictive temperatures. In contrast, the 2.4 Kb transcript was present at an elevated level in RNA prepared from five out six blocked cdc mutants tested. In RNA obtained from wild type or cdc 2 mutant cells incubated at the restrictive temperature this 2.4 Kb transcript was present at a greatly reduced level.

CHAPTER 7Genetical Analysis of the cdc 22 Locus1 cdc 22 - C11 - Another Allele of the cdc 22 Locus

During the course of this work, seven cdc⁻ temperature sensitive mutants were isolated independently by Dr Jim Creanor with an execution point at or before DNA synthesis in S. pombe. (J. Creanor; Personal Communication).

As an initial test for linkage with the cdc 22 locus, each mutant in an h⁻ genetic background was crossed to a cdc 22 - M45 h⁺ mutant strain. The progeny from each cross were streaked out on yeast extract agar containing Phloxin B and incubated at 35°C in order to detect wild type recombinants. (Nurse et al, 1976). In most cases, a high frequency of recombinants was observed indicating that the mutant strain mapped in an unlinked gene. However, in the cross involving the strain C11, a low frequency of cdc⁺ strains was observed indicating a mutation in the same or a closely linked gene.

To distinguish between the above two possibilities a complementation test was carried out by constructing a stable cdc 22 - M45/C11 diploid as described in Materials and Methods. Out of five independent stable diploids tested no growth was observed at 35°C. This suggested that the cdc⁻ mutation present in the C11 strain was in the cdc 22 gene. An alternative explanation which was not checked was that the C11 mutation was dominant to wild type. However, this was thought unlikely as the C11 cdc⁻ phenotype had already been shown to be linked to the cdc 22 locus.

To determine if the cdc 22 - C11 mutation was a different allele

than cdc 22 - M45 the level of recombination between the two mutations was measured by free spore analysis. The recombination frequencies obtained are given in Table 7.1. The recombination frequency obtained between the cdc 22 - C11 and cdc 22 - M45 mutant strains was a hundred times greater than that found in the control homoallelic crosses. This data suggests the cdc 22 - C11 mutation is a different allele of the cdc 22 gene to the cdc 22 - M45 mutation. The frequency of recombination between cdc 22 - C11 and cdc 22 - M45 is consistent with intragenic recombination frequencies previously observed in S. pombe (Nurse and Thuriaux, 1930).

2 Allocation of the cdc 22 gene to a linkage group

A cdc 22 - M45 leu 1.32 ade 6.704 h⁻ strain was crossed to a mat 2. B102 lys 1.131 strain. The resulting diploids were selected by complementation on EM + Phloxin. Single diploid colonies were incubated overnight at 25°C as a patch on yeast extract agar containing 10 µg/ml of benomyl (Gift from Dr. D. Roy, Dept of Microbiology, University of Edinburgh) to induce mitotic haploidisation. A cell suspension was then plated out on yeast extract plus Phloxin at 25°C, and sixty haploid colonies identified by their pale pink colony appearance. These haploid colonies were analysed for cosegregation of the cdc 22 marker with lys 1 (chromosome 1), leu 1 (chromosome 2) or ade 6 (chromosome 3).

The results obtained are shown in Table 7.2. The data is consistent with the cdc 22 gene being situated on chromosome 1.

One surprising result was the low number of ade⁻ colonies obtained as if some selection against ade⁻ colonies occurred during haploidisation. This is because the complex media used, yeast extract,

Table 7.1Recombination Frequencies between cdc 22-M45 and cdc 22 - C11

STRAIN	cdc 22 - M45 h ⁻	cdc 22 C11 h ⁻
<u>cdc</u> 22 - M45 h ⁺	0.2	167
<u>cdc</u> 22 C11 h ⁺	62	0.6

Frequencies are given in wild type recombinants/10⁶ spores.

Table 7.2

		<u>lys 1</u>		Total	
		+	-		
<u>cdc 22</u>	+	0	34*	34	
	-	21*	0	21	$\chi^2 = 55$
Total		21	34	55	

		<u>leu 1</u>		Total	P < 0.01 linkage
		+	-		
<u>cdc 22</u>	+	12*	23	35	
	-	11	11*	22	$\chi^2 = 2.12$
Total		23	34	57	

		<u>ade 6</u>		Total	P > 0.05 no linkage
		+	-		
<u>cdc 22</u>	+	34*	2	36	
	-	21	0*	21	$\chi^2 = 2.12$
Total		55	2	57	

				Total	P > 0.05 no linkage

* indicates parental genotype

contains limiting amounts of adenine (Gutz et al, 1974).

3 Summary

Another allele of the cdc 22 gene designated cdc 22 - C11 has been isolated.

The cdc 22 locus has been shown by haploidisation analysis to be situated on chromosome I.

CHAPTER 3Time of the cdc 22 Gene Product Function and its Relation to S phase1 Introduction

Nasmyth and Nurse (1981) characterised a cdc 22 - M45 strain by carrying out two types of experiments. First, no DNA synthesis occurred in a synchronised culture of cdc 22 - M45 cells after shift to 36°C. Secondly, cdc 22 - M45 cells arrested with hydroxyurea at the permissive temperature were capable of a further round of division if shifted to the restrictive temperature in the absence of the drug. As hydroxyurea specifically blocks S phase in S. pombe (Mitchison and Creanor, 1971) this suggested that the cdc 22 gene product was required for the initiation of DNA synthesis.

The following chapter describes the further characterisation of cdc 22 mutant cells. Strains carrying the mutant allele 22-M45 or 22-311 were investigated. First, the response of both strains to a shift from the permissive to the restrictive temperature was investigated in a wee 1⁺ and wee 1 allele. The wee 1 mutants are altered in both cell size at division and in the relative duration of the G₁ and G₂ phases (Nurse, 1975; Nurse and Thuriaux, 1977; Fantes and Nurse, 1973; discussed in the Introduction, Chapter 1). An analysis of the results allowed the transition point of the cdc 22 gene product to be calculated for each mutant strain. The transition point is the point in the cell cycle after which a shift from the permissive to the restrictive temperature can no longer prevent the completion of the cell cycle (Pringle, 1978). Although caution must be used when interpreting transition point data (discussed in detail in Pringle, 1978) they can give an estimate of the time in the normal cell cycle when the wild

type gene product completes its function.

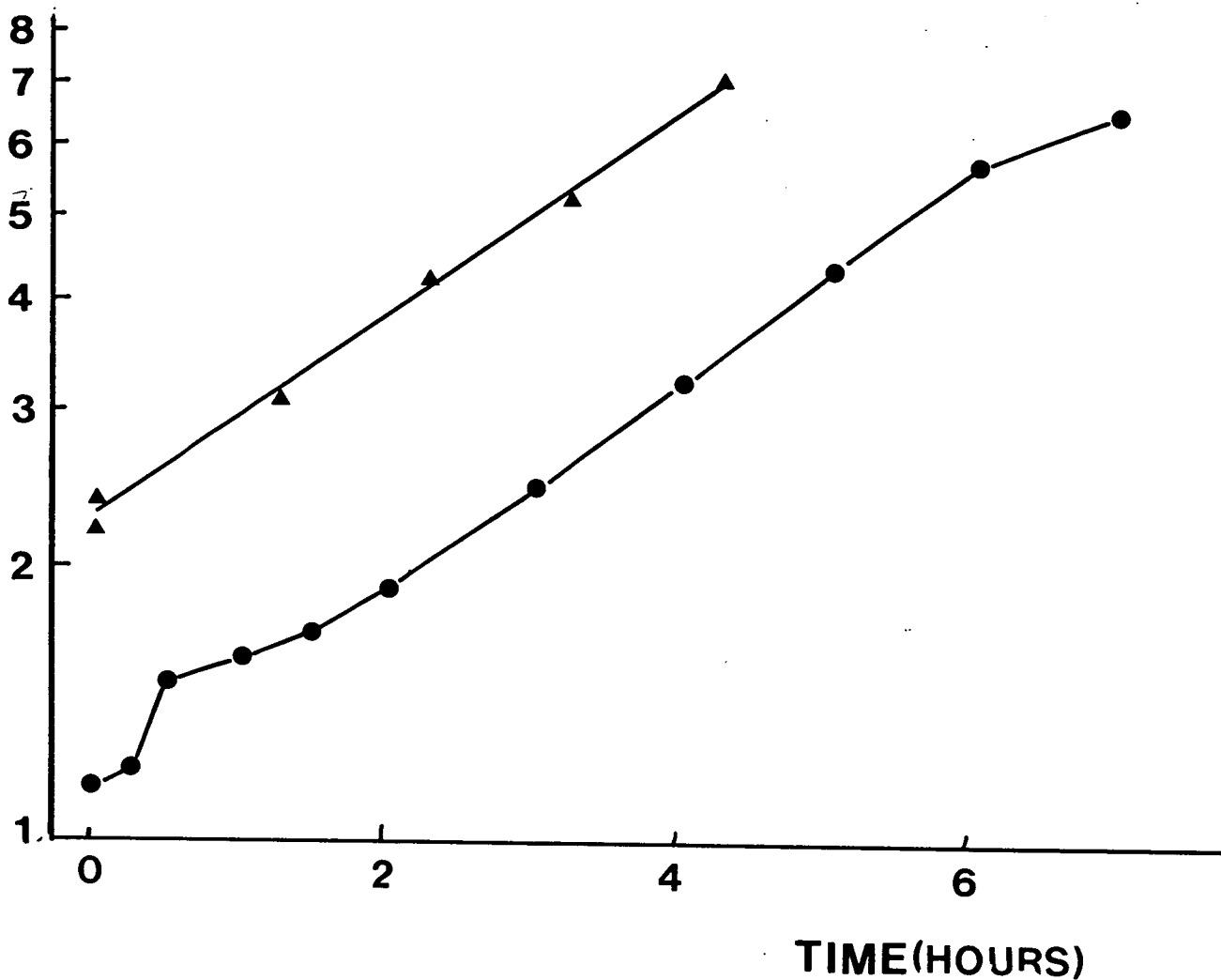
Secondly, the timing of S phase was estimated in cdc 22 - 145, cdc 22 - 145 wee 1.6, wee 1.6 and 972 wild type cells at the permissive temperature by blocking cells with the chemical inhibitor hydroxyurea. The transition point of hydroxyurea was calculated from the fractional increase in cell number after the drug was added to an asynchronous exponentially growing culture.

2 Response of 972 Wild Type Cells to Temperature Shift

A wild type exponentially growing culture was shifted from 25°C to 36°C. Two parameters of the culture were measured after shift to the higher temperature. Cell number was determined as detailed in Materials and Methods. Cell growth was estimated by the increase in turbidity of the culture as described in Materials and Methods.

After shift to 36°C wild type cells continued to divide for approximately 30 minutes (Figure 3.1). This was followed by a short plateau in cell number which lasted for about 45 minutes. Cell division then resumed at an increased rate for the duration of the experiment. The transient plateau in cell number has been previously reported (Nurse, 1975) and results from a temporary inhibition of nuclear division following the shift to the higher temperature.

The turbidity of the culture increased continuously after temperature shift up indicating that growth was also continuous.

Figure 8.1 Response of Wild Type Cells to Shift from 25°C to 36°C

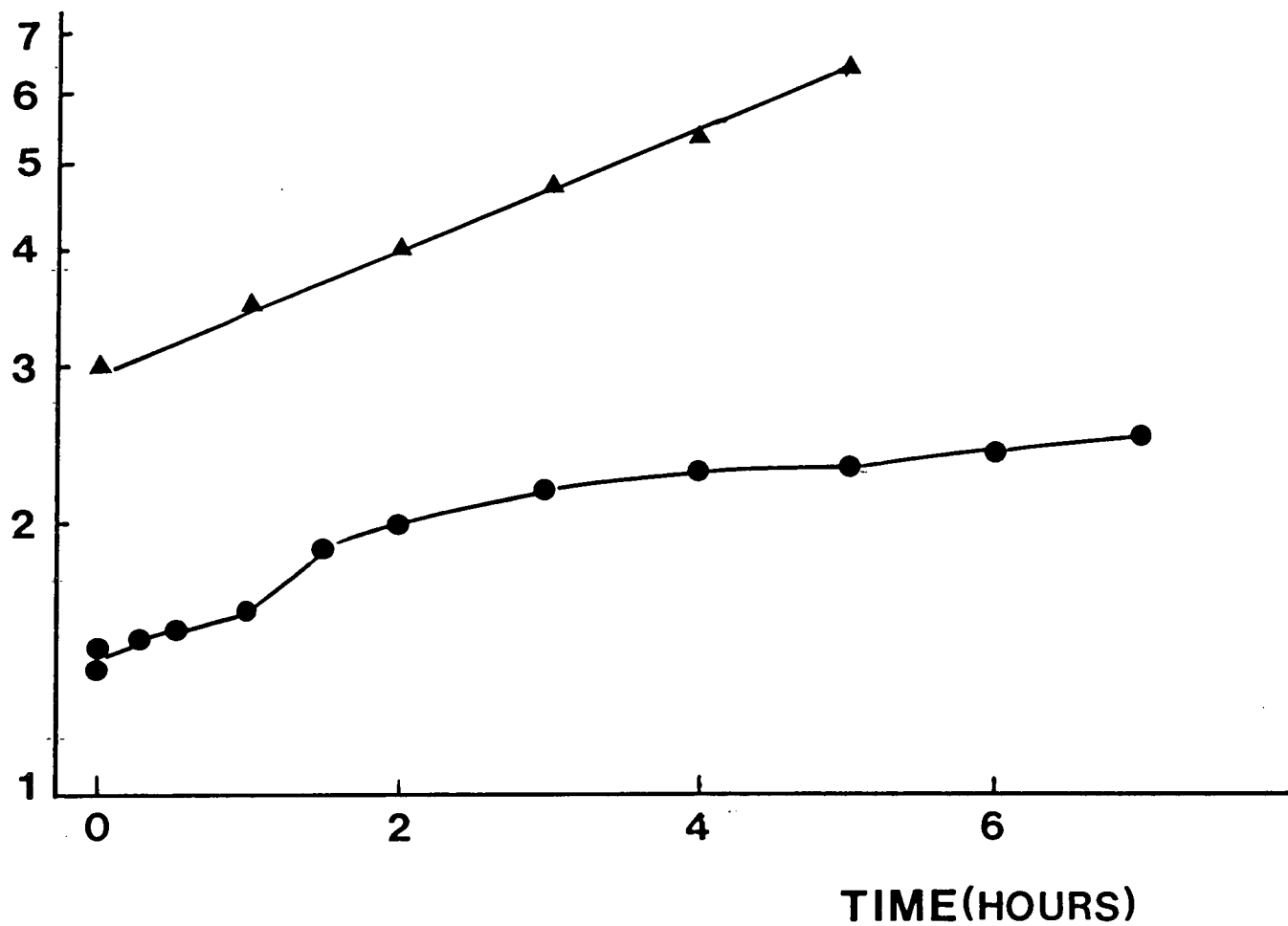
At time 0 an exponentially growing asynchronous culture of 972 wild type cells in EMM3 was shifted from 25°C to 36°C. Samples were taken at the times shown to monitor cell number (●) and turbidity (▲) of the culture as described in Materials and Methods. Both parameters were plotted as arbitrary units on a log scale against time. For cell number 1 unit is equivalent to 4×10^5 cells ml^{-1} and for turbidity 1 unit is equivalent to 0.1 O.D. units.

3 Response of the cdc 22 Strains to Temperature Shift up

The response to a shift from 25°C to 36°C was investigated in cdc 22 - M45, cdc 22 - M45 wee 1.6, cdc 22 - C11 and cdc 22 - C11 wee 1.6 (shown in Figures 3.2 to 3.5 respectively). All the mutant strains showed a similar response to the shift in temperature which was different to that observed with wild type cells. Cell number increased for a time, between 2 and 4 hours depending on the mutant strain, after which cell number plateaued for the remainder of the experiment. In marked contrast, the turbidity increased exponentially.

The timing of the transition point for the cdc 22 gene product was determined for each strain from the fraction of cells able to complete division following shift to the restrictive temperature as detailed in Nurse et al (1976). The transition points calculated from the data in Figures 3.2 to 3.5 are presented in Table 3.1. The transition point of the cdc 22 - C11 strain at - 0.25 is consistent with the cdc 22 gene product being required for the initiation of DNA replication as S phase occurs at 0 in the normal S. pombe cell cycle (Nasmyth et al, 1979). This transition point is similar to that observed for cdc 20 - M10, which is also thought to be defective in the initiation of DNA replication (Nasmyth and Nurse, 1981). The transition point of the cdc 22 - C11 wee 1.6 strain is 0.1 of a cycle later than in a wee 1⁺ strain. This is broadly consistent with S phase being delayed by 0.2 of a cycle in wee 1 mutant cells (Nasmyth et al, 1979).

In contrast, the transition point of the cdc 22 gene product in the cdc 22 - M45 strain was calculated to be 0.28. This is substantially later than S phase (at 0.0) in wild type cells. In addition, surprisingly little difference was found between the transition points of the

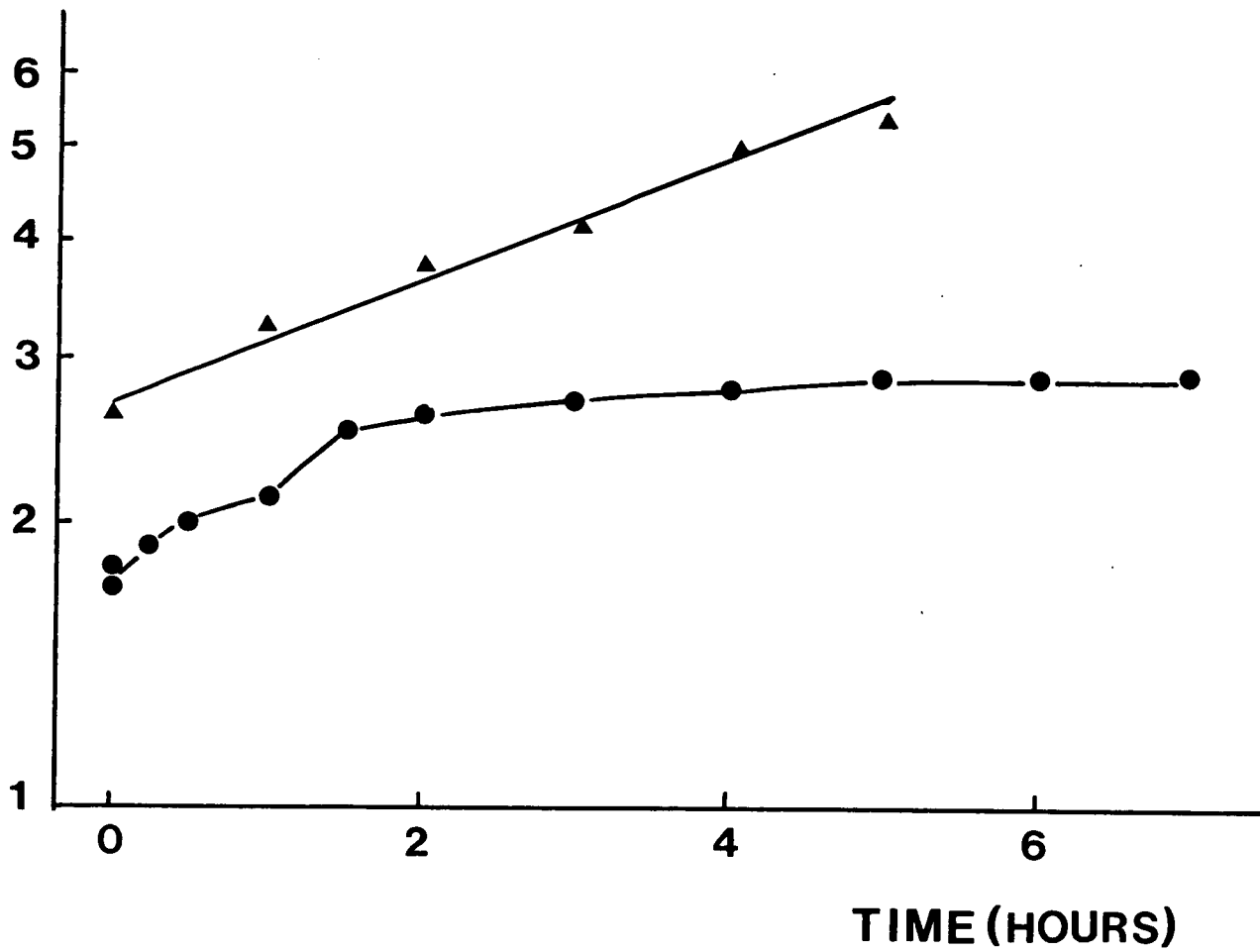
Figure 8.2 Response of cdc 22 - M45 Cells to Shift to 36°C

Description as for Figure 8.1.

Cell number ●

Turbidity ▲

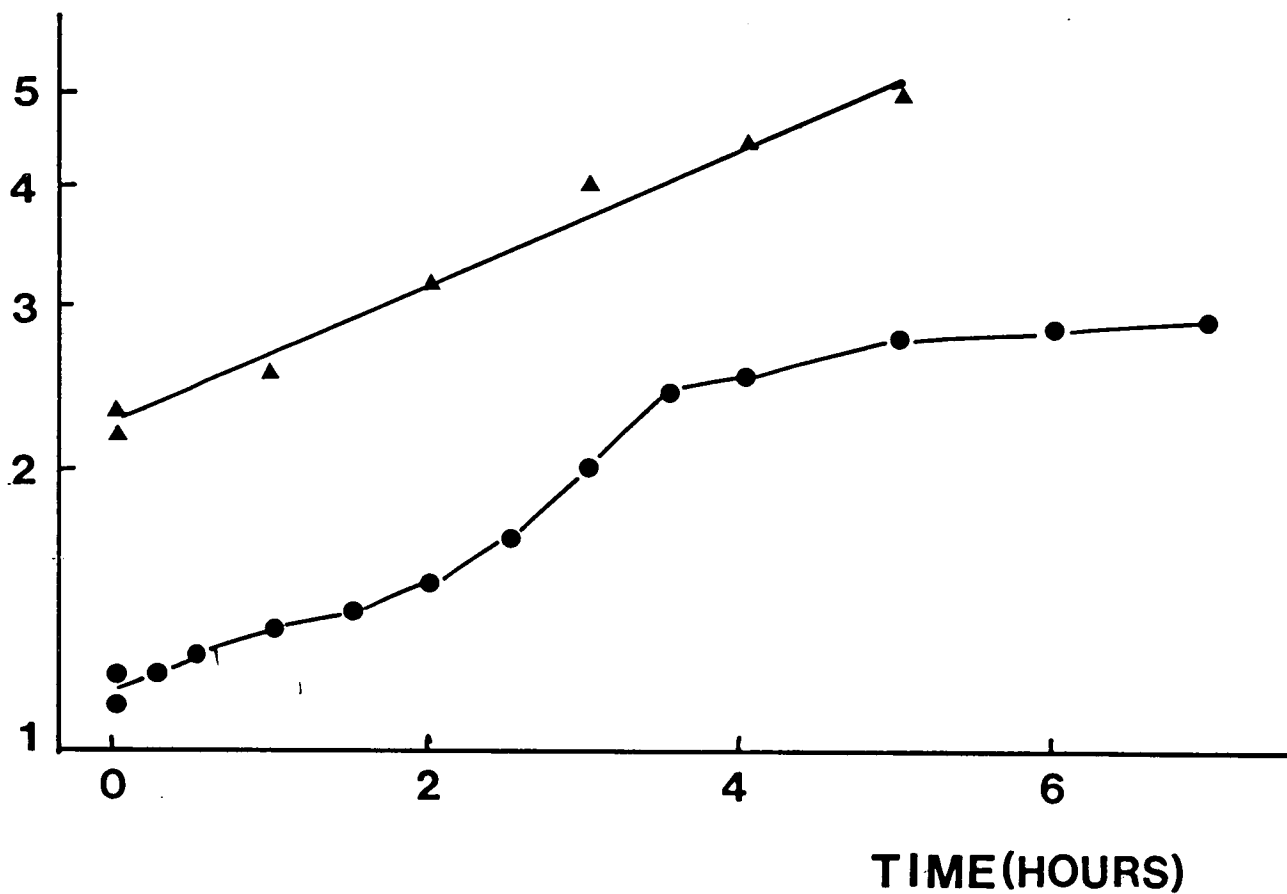
Figure 8.3 Response of cdc 22 - M45 wee 1.6 cells to a Shift
from 25°C to 36°C



Description as for Figure 8.1.

Cell number ●

Turbidity ▲

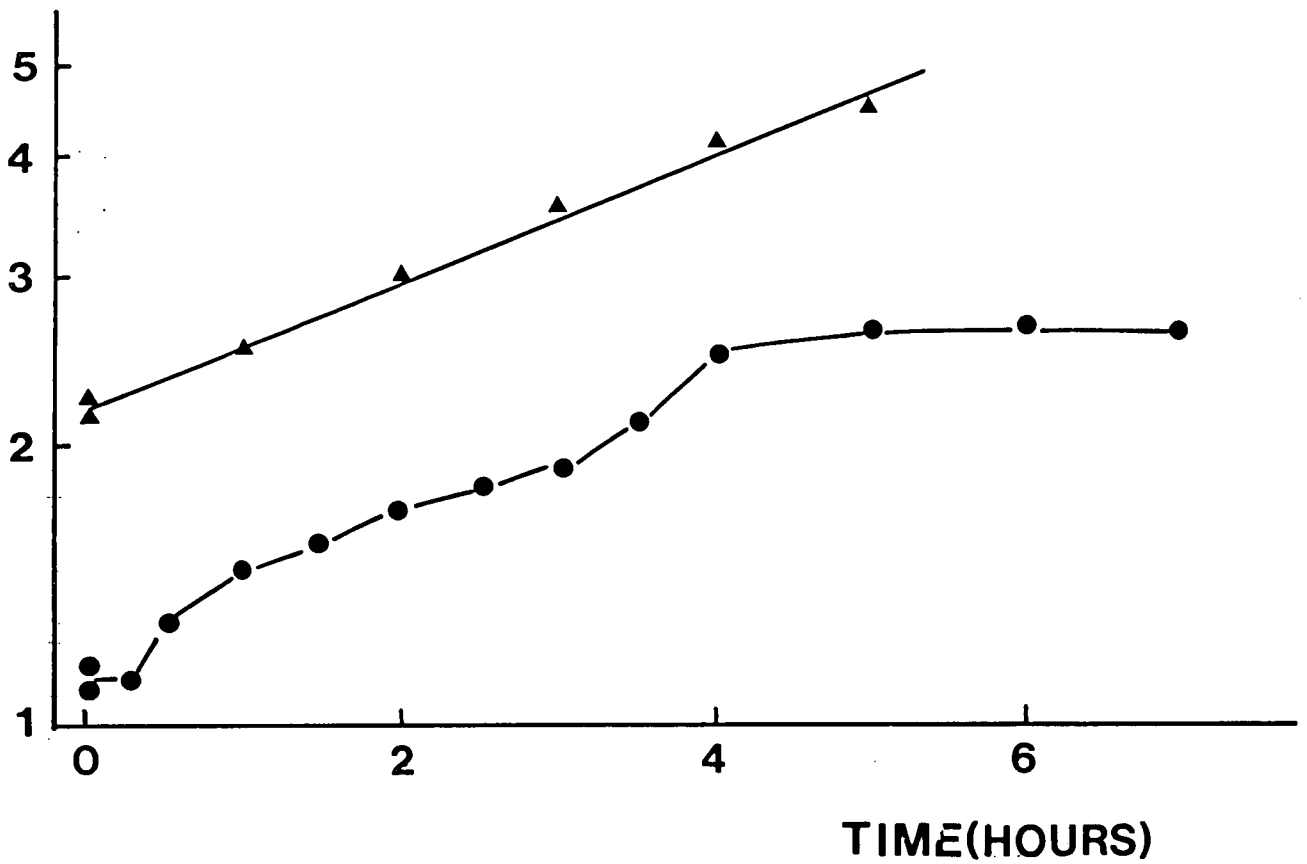
Figure 8.4 Response of cdc 22 - C11 Cells to-a Shift from 25°C to 36°C

Description as for Figure 8.1.

Cell number ●

Turbidity ▲

Figure 8.5 Response of cdc 22 - C11 wee 1.6 Cells of a Shift from
25°C to 36°C



Description as for figure 8.1.

Cell number ●

Turbidity ▲

Table 8.1 Transition Points of Different cdc 22 Strains

STRAIN ^a	TIME (MINS)	CELL NO ^b ($4 \times 10^5 \text{ ml}^{-1}$)	FRACTIONAL CELL ^c NO. INCREASE	TRANSITION ^d POINT
<u>cdc 22-M45</u>	0	1.43	1	-
	240	2.36	1.65	0.28
	420	2.54	1.78	0.17
<u>cdc 22-M45 wee 1.6</u>	0	1.67	1	-
	180	2.71	1.62	0.30
	420	2.84	1.65	0.23
<u>cdc 22-C11</u>	0	1.16	1	-
	300	2.77	2.39	-0.25
	420	2.86	2.46	-0.30
<u>cdc 22-C11 wee 1.6</u>	0	1.13	1	-
	300	2.51	2.22	-0.15
	420	2.64	2.34	-0.22

- a. The data for cdc 22-M45 are shown in Figure 8.2, cdc 22-M45 wee 1.6 in Figure 8.3, cdc 22-C11 in Figure 8.4 and cdc 22-C11 wee 1.6 in Figure 8.5.
- b. Cell number was estimated from a 0.1 ml sample counted on a coulter particle counter. The values given are averages of two counts.
- c. The fractional cell number increase was estimated from the following equation.

$$\text{Fractional Cell Number Increase} = N/N_0$$

N = Cell number at the plateau in cell number.

No = Initial cell number when the culture was shifted from the permissive to the restrictive temperature at time 0.

- d. The transition point was estimated using the equation of Howell et al, 1975.

$$\text{Transition point} = 1 - \frac{\log_e (N/N_0)}{\log_e 2}$$

cdc 22 gene product in a cdc 22 - M45 strain, at 0.28, and cdc 22 - M45 wee 1.6 strain, at 0.3. One possible explanation for the unexpected results with the cdc 22 - M45 cells is that S phase is delayed in this strain. This could be due to the cdc 22 gene product being only partially active at the permissive temperature in a cdc 22 - M45 strain. Partial activity of the cdc gene product in mutant strains at the permissive temperature has been reported. For example, Nasmyth (1977) has demonstrated that cdc 17 mutants of S. pombe, which are defective in DNA ligase, have low DNA ligase activity at the permissive temperature. This partial activity will only affect cell cycle behaviour if the activity becomes limiting. In the case of the mutant cdc 17 strains even though the mutant ligase is only partially active at the permissive temperature no cell cycle distortions are observed presumably because the ligase enzyme is present in considerable excess (Nasmyth, 1977).

Experiments to test the hypothesis that S phase is delayed in a cdc 22 - M45 strain are described in the next section.

4 Response of 972, wee 1.6, cdc 22 - M45 and cdc 22 - M45 wee 1.6 cells to Hydroxyurea

As described in the last section the transition point of 0.28 for cdc 22 - M45 is contrary with the cdc 22 gene product being required for the initiation of DNA synthesis. A possible explanation could be that the S phase is delayed in a cdc 22 - M45 strain. The time in the cell cycle at which S phase occurred was investigated in 972 wild type, wee 1.6, cdc 22 - M45 and cdc 22 - M45 wee 1.6 strains. An estimate of the timing of S phase in each strain was obtained by calculating the transition point for hydroxyurea-induced cell cycle

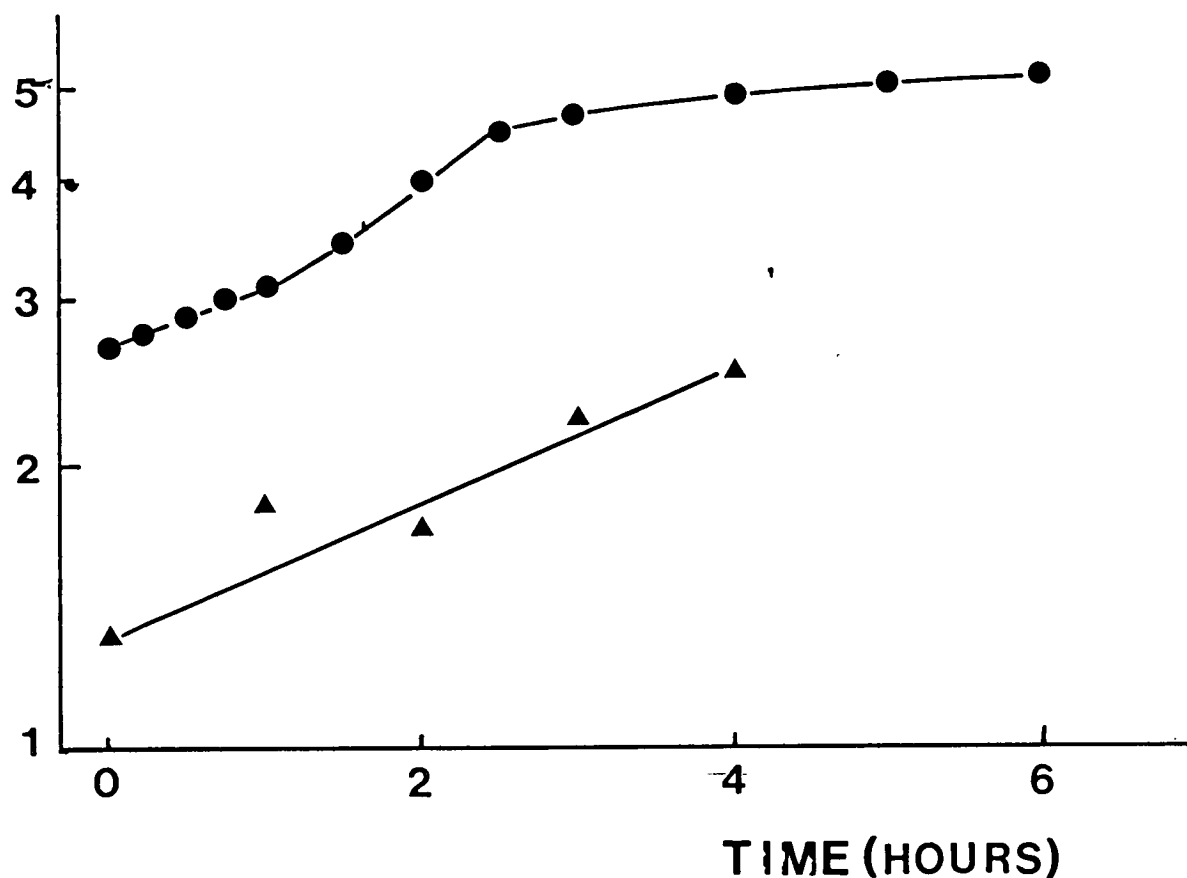
arrest.

Hydroxyurea was added to a final concentration of 12 mM to an early log phase asynchronous culture growing at 25°C. Cell number and cell growth were measured. The response of 972, wee 1.6, cdc 22 - M45 and cdc 22 - M45 wee 1.6 strains to incubation in the presence of 12 mM hydroxyurea is shown in Figures 3.6 to 3.9 respectively. The transition points for hydroxyurea were calculated as before from the residual cell number increase and are shown in Table 3.2.

The hydroxyurea transition point of 0.12 for 972 cells is slightly later than the timing of S phase at 0.0, in the normal S. pombe cell cycle. The transition point of 0.46 for hydroxyurea in wee 1.6 cells is close to the time at which S phase occurs, at 0.31, in the wee 1 cell cycle (Nasmyth et al, 1979). However, the transition point of 0.42 observed for hydroxyurea in a cdc 22 - M45 strain is almost the same value as that obtained in a cdc 22 - M45 wee 1.6 strain at 0.4.

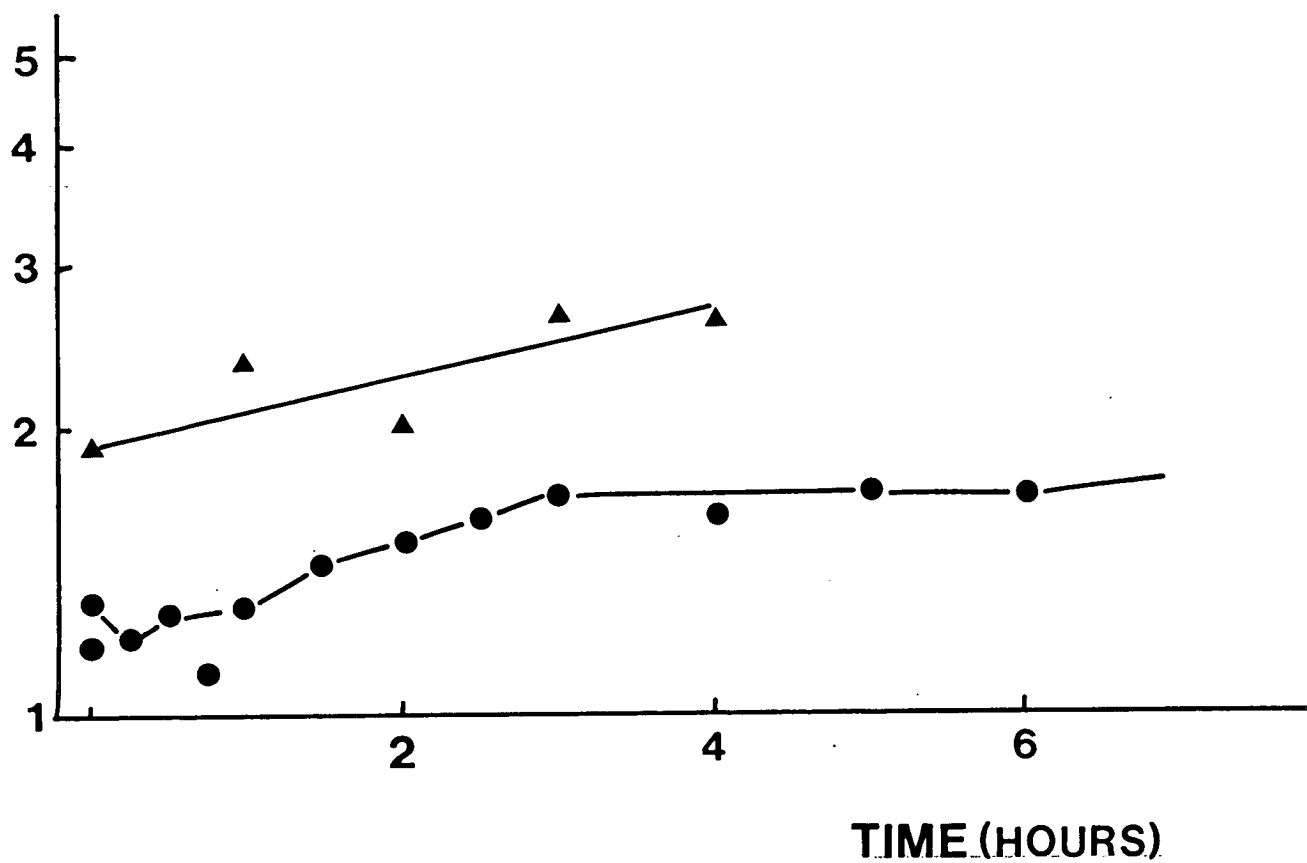
Thus, the defective cdc 22 - M45 gene product seems to have no effect on the timing of S phase in a wee 1 strain as the transition points of hydroxyurea obtained with wee 1.6 and cdc 22 - M45 wee 1.6 are similar (0.46 and 0.4 respectively). In contrast, in a wee 1⁺ strain the cdc 22 - M45 gene product causes a substantial delay in the time at which S phase occurs as the hydroxyurea transition points are 0.12 for wild type cells and 0.42 for a cdc 22 - M45 strain. It has been demonstrated that a minimum time exists for the completion of S and G₂ phases of the cell cycle (Fantès and Nurse, 1973; Nasmyth, 1979). In wee 1.6 cells S phase is delayed by the requirement that a cell must attain a critical mass before DNA replication can take place (Nurse, 1975; Nurse and Thuriaux, 1977). Perhaps the similar transition points of hydroxyurea observed with wee 1.6 and cdc 22 -

Figure 8.6 Response of 972 Cells to Incubation in the Presence of 12 mM Hydroxyurea



At time 0, hydroxyurea was added to a final concentration of 12 mM to an exponential 972 asynchronous culture in EMM3 at 25°C. Samples were taken, as before, to monitor cell number (●) and turbidity (▲). Both parameters were plotted on a log scale against time. For cell number 1 unit is equivalent to 4×10^5 cells ml^{-1} and for turbidity 1 unit is equivalent to 0.1 O.D. units.

Figure 8.7 Response of wee 1.6 Cells to Incubation in 12 mM Hydroxyurea

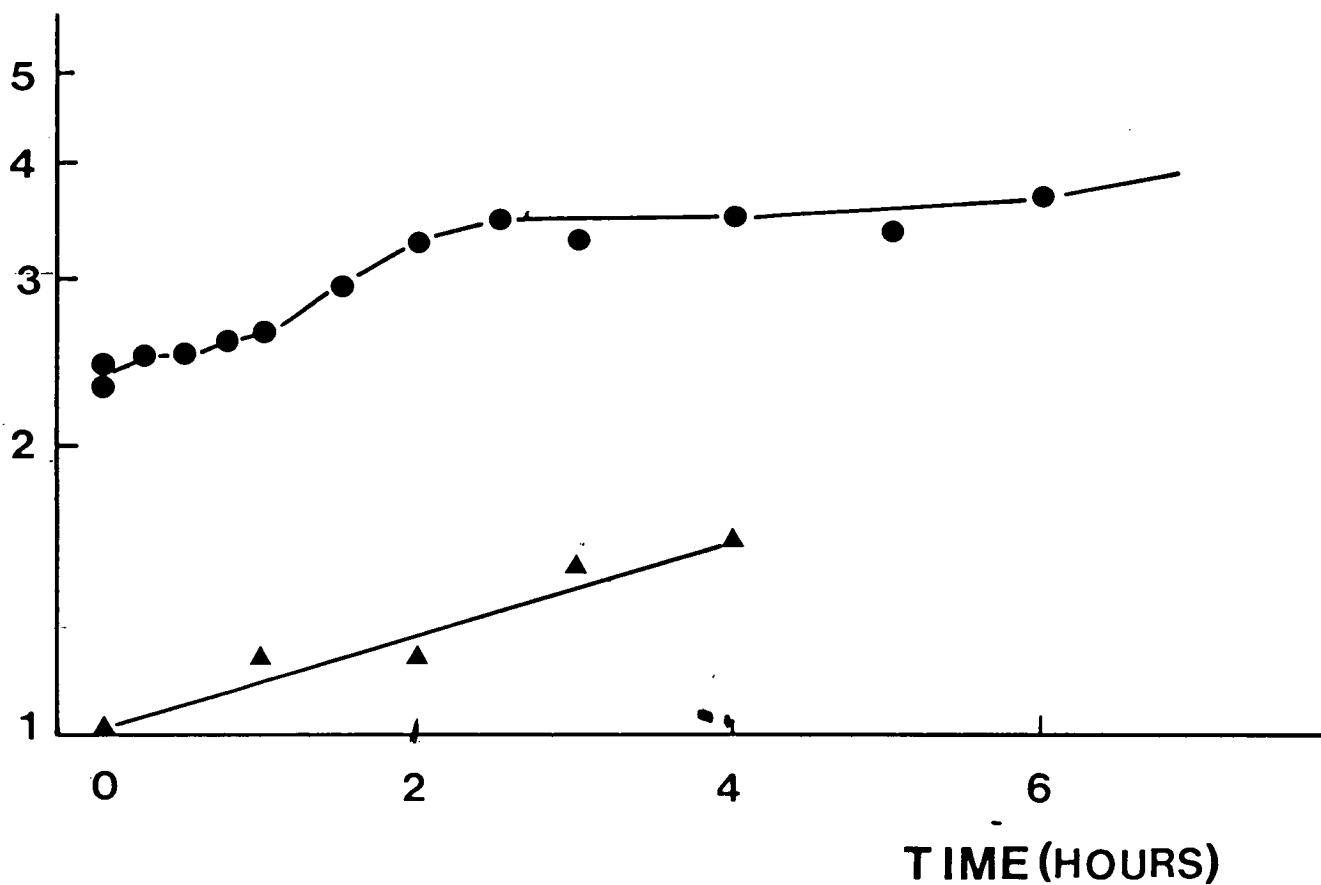


Description same as for Figure 8.6.

Cell number ●

Turbidity ▼

Figure 8.8 Response of cdc 22 - M45 Cells to Incubation in 12 mM Hydroxyurea

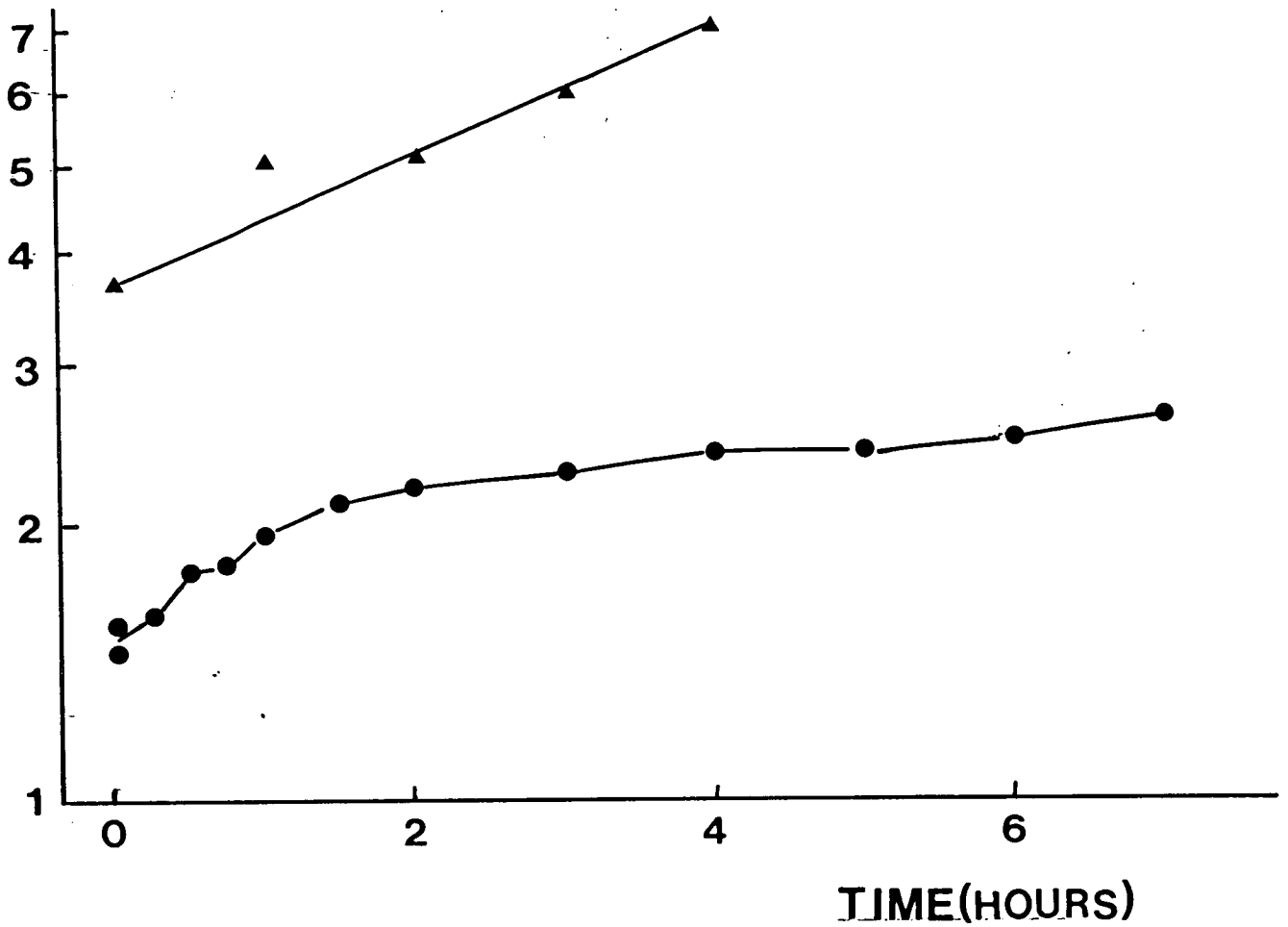


Description same as for Figure 8.6.

Cell number ●

Turbidity ▼

174
Figure 8.9 Response of cdc 22 - M45 wee 1.6 Cells to Incubation in 12 mM Hydroxyurea



Description same as for Figure 8.6.

Cell Number ●

Turbidity ▼

M45 wee 1.6 strains is because the limit to which S phase can be delayed has been reached.

5 Conclusion

The transition point of the cdc 22 gene product was calculated for cdc 22 - C11 and cdc 22 - M45 strains. The values obtained differed substantially from one another. The transition point obtained for the cdc 22 - C11 strain at -0.25 is before when S phase occurs in wild type cells. This value is also similar to the transition point of the cdc 20 gene, which is also required for the initiation of DNA synthesis.

In contrast, the transition point of 0.28 for the cdc 22 gene product in a cdc 22 - M45 strain is appreciably later than when S phase would occur in the normal cell cycle. Evidence based on the transition point of hydroxyurea in a cdc 22 - M45 suggests that S phase is delayed in this strain. Thus of the two transition points of the cdc 22 gene product, the value obtained in a cdc 22 - C11 strain would be expected to give a more reliable estimate of when the cdc 22 product is required during the wild type cell cycle. This provides further evidence that the cdc 22 gene product is involved in the initiation of DNA replication.

Table 8.2 Transition Points of Hydroxyurea in Different Strains

STRAIN ^a	TIME (MINS)	CELL NO ($4 \times 10^5 \text{ ml}^{-1}$)	FRACTIONAL CELL NO. INCREASE	TRANSITION POINT
972	0	2.68	1	-
"	240	4.92	1.84	0.12
"	420	5.13	1.91	0.06
<u>wee</u> 1.6	0	1.2	1	-
"	300	1.71	1.42	0.46
"	420	1.76	1.47	0.45
<u>cdc</u> 22-M45	0	2.42	1	-
"	150	3.62	1.50	0.42
"	420	4.00	1.65	0.26
<u>cdc</u> 22-M45 <u>wee</u> 1.6	0	1.56	1	-
"	240	2.37	1.52	0.4
"	420	2.62	1.68	0.25

- a. The data for the 972 culture are shown in Figure 8.6, wee 1.6 in Figure 8.7, cdc 22-M45 in Figure 8.8 and cdc 22-M45 wee 1.6 in Figure 8.9.

The cell number, fractional cell number increase and transition points were estimated as described in Table 8.1.

CHAPTER 9 Discussion1 Identification of the cdc 22 Gene Product(i) Is the cdc 22 Gene Product NDPK?

As described in the Introduction the cdc 22 gene was, at the start of this work, thought to be the structural gene for the DNA precursor enzyme nucleoside diphosphokinase (NDPK). This enzyme is one of two in S. pombe that has been found to show a periodic stepwise doubling of activity during the cell cycle. The initial aim of this project was to clone the cdc 22 gene to use as a hybridisation probe to assay for the cdc 22 transcript. This cdc 22 specific probe would be used to investigate if the periodic step in NDPK activity was also paralleled by a periodic change in the level of the cdc 22 transcript.

The original evidence for NDPK being encoded by the cdc 22 gene was based on work by Dickinson (1981) on the biochemical characterisation of the cdc 22 - M45 mutant strain. Nucleotide pools were analysed from a cdc 22 - M45 strain that had been incubated at the restrictive temperature. The concentration of dTTP was found to be reduced while the concentration of dTDP increased compared to wild type cells. Dickinson's interpretation of the differences in nucleotide pool levels was that the enzyme responsible for catalysing the conversion of dTDP to dTTP, NDPK, was defective in the cdc 22 - M45 strain.

To test this hypothesis crude cell extracts prepared from cdc 22 - M45 and wild type cells were assayed for NDPK activity. When assayed at the restrictive temperature NDPK activity from cdc 22 - M45 cells was found to be 1.6 times less than that obtained from wild type cells. To test whether this small difference in activity was the result of a structural alteration in the NDPK of

cdc 22 - M45 cells, the K_m values of NDPK from cdc 22 - M45 and wild type cells were estimated. When estimated at the restrictive temperature the K_m values obtained for NDPK from cdc 22 - M45 cells was eleven times greater as that obtained for wild type cells. Dickinson concluded that the conditional lethal phenotype of the cdc 22 - M45 strain was the result of a temperature sensitive defect in NDPK, leading to a defect in DNA synthesis and hence cell division.

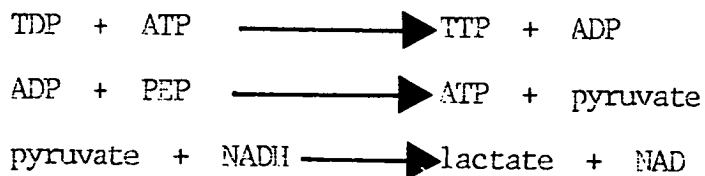
However a number of observations conflict with this conclusion. In S. cerevisiae, the cdc 8 and cdc 21 mutant strains have been shown to be defective in thymidylate kinase and thymidylate synthetase respectively (Sclafani and Fangman, 1984; Game, 1976). Both these enzymes, like NDPK, are involved in DNA precursor metabolism. Physiological characterisation of these mutants by Hartwell (1976) demonstrated that in reciprocal shift experiments the cdc 8 and cdc 21 gene product function was interdependent with the hydroxyurea sensitive step. This suggested that both these DNA precursor mutants were required for DNA chain elongation. In contrast, Nasmyth and Nurse (1981) showed that in S. pombe the cdc 22 gene product was required before the block imposed by hydroxyurea. This was consistent with the cdc 22 - M45 strain being deficient in the initiation of DNA replication rather than chain elongation.

The transition point is the time in the cell cycle beyond which a shift from the permissive to the restrictive temperature can no longer prevent a mutant cell from successfully completing a cell cycle (Nurse et al, 1976). The transition point estimated for the cdc 22 - M45 allele, at 0.28, is consistent with a defect in chain elongation. However evidence presented in Chapter 8 based on the transition point of hydroxyurea in a cdc 22 - M45 strain argues that the

cell cycle is distorted in this mutant. However, the transition point calculated for the cdc 22 - C11 allele at - 0.2 is similar to other S. pombe cdc mutants such as cdc 10 and cdc 20 which are defective in the initiation of DNA synthesis (Chapter 8; Nasmyth and Nurse, 1981; Nurse et al, 1976).

Indirect evidence described in this thesis also suggests that the cdc 22 gene is unlikely to be the structural gene for NDPK. The cloned cdc 22 gene was found to code for a mRNA of 3.3 Kb in size. From the size of this transcript the molecular weight of the polypeptide coded by it can be estimated provided certain assumptions are made. For example, it is assumed the 3.3 Kb transcript detected is the final processed form, the transcript does not code for tandem copies of the same polypeptide and no large untranslated segments are present on the mRNA. If the average weight of an amino acid is taken to be 110 the polypeptide coded by the cdc 22 gene would have an approximate molecular weight of 120,000. NDPK has been purified to homogeneity from S. cerevisiae. The enzyme is composed of six identical sub units of molecular weight 17,000 (Palmieri et al, 1973). The results of preliminary experiments on NDPK purified from S. pombe suggests that the enzyme has a similar structure and molecular weight to that found in S. cerevisiae (Dr. L Henley, Personal Communication). Thus the cdc 22 codes for a polypeptide whose estimated molecular weight is six times that of the NDPK polypeptide monomer.

However, the strongest evidence against cdc 22 coding for NDPK has come from a re-examination of the NDPK activity in cdc 22 - M45 cells using a more direct assay than that used by Dickinson (Dr J. Creanor, Personal Communication). Dickinson's assay for NDPK consisted of coupling the following reactions:



The formation of TTP was ultimately coupled to the disappearance of NADH, which was measured by a fall in the absorbance at 360 nm. In contrast the radioactive assay used by Creanor measured the transfer of ^{32}P from γ labelled ATP to TDP. Thus the appearance of ^{32}P - TTP was measured directly. The radioactive assay was found to be more reliable than measuring the fall in NADH after a series of two coupling reactions (Dr J. Creanor, Personal Communication). Using the radioactive assay Creanor found no difference in the activity of NDPK from cdc 22 - M45 or cdc 22 - C11 cell extracts compared to wild type cells, even when NDPK was assayed at as high a temperature as 60°C.

Although the most simplest explanation of the pool data discussed earlier is that cdc 22 - M45 cells are defective in NDPK, a number of observations now conflict with this conclusion. On balance it now seems likely that the cdc 22 gene enzyme relationship may have been mistaken.

(ii) Possible Involvement of the cdc 22 Gene Product in Histone Transcription

A possible clue to the function of the cdc 22 gene product has emerged from an investigation of the core histone H2A transcript levels during the cell cycle. Histones are small basic proteins involved in packaging DNA to form the chromatin structure (Alberts et al, 1983). In wild type S. pombe cells the level of histone H2A mRNA is cell cycle regulated reaching a peak during S phase (Aves et al, 1985; Chapter 5, figure 5.8). Additional experiments by Nurse and fellow

workers (Dr P. Nurse, Personal Communication) examined the histone H2A levels in blocked "synchronous cultures" of various cdc mutant cells. In these experiments a synchronous culture of a cdc mutant strain was prepared by size selection from cells grown at the permissive temperature. The synchronous culture was shifted to the restrictive temperature and the histone transcript levels followed for a period equivalent to two cell cycles. In cdc 10 cells, which arrest in G₁ (Nurse et al, 1976), the histone transcripts never rose above a basal level. In blocked "synchronous cultures" of cdc 20 and cdc 17 mutant cells, which arrest at the initiation and termination of S phase respectively (Nasmyth and Nurse, 1981), the histone transcript levels peaked once and then remained at a basal level. However for a blocked cdc 22 synchronous culture, which also arrests at the initiation of DNA synthesis, an unusual pattern of histone H2A transcript levels was observed. In a blocked cdc 22 culture the histone H2A transcript levels continued to fluctuate periodically peaking twice during the experiment. This pattern was similar to that obtained with unblocked wild type cells. This striking observation suggests that the level of the histone H2A mRNA continues to fluctuate periodically even though cell cycle progress is blocked in these cells.

In S. cerevisiae it has been clearly demonstrated that the regulation of histone transcript levels occurs by two distinct mechanisms (Hereford et al, 1982; also see next section). The first is by periodic transcription of the histone genes, while the second is exerted postranscriptionally and acts on the stability of histone mRNA. It seems likely that the regulation of H2A levels in S. pombe occurs in an analagous manner. The following argument will concentrate on how the cdc 22 gene product may be involved in the periodic transcription control.

In a blocked cdc 10 culture the H2A transcript levels never rise above a basal level. This suggests that the cdc 10 cells arrest before the point in the cell cycle to where histone H2A transcription initiates. In cdc 20 and cdc 17 blocked cells the level of H2A mRNA rises to a peak, falls but is not reinitiated. This implies that both the initiation and termination of histone H2A transcription occurs before the points at which the cdc 20 and cdc 17 mutants arrest. However in cdc 22 blocked cells the H2A transcript continues to rise and fall periodically at a similar time to unblocked wild type cells. The unexpected observation that the level of histone H2A mRNA continues to behave periodically in cdc 22 arrested cells suggests that H2A transcription has the potential to show continued periodicity in the absence of cell cycle progress. If this is the case it would indicate that the initiation of histone transcription may be controlled by an oscillator that is independent of the nuclear division cycle. Furthermore, the failure of reinitiation of histone H2A transcription in cdc 20 and cdc 17 blocked cells suggests that the cdc 22⁺ gene product inhibits the reinitiation of histone H2A transcription. Thus when the cdc 22 gene product itself is defective, as in a blocked cdc 22 culture, this repression is removed.

A simple explanation of the histone H2A mRNA pattern in different blocked S. pombe cdc mutant is that a positive and negative element act on the initiation of histone H2A transcription. The nature of the positive element is unknown but it would be expected to continue to oscillate in cells defective in cell cycle progress. The observations discussed for the cdc 22 blocked cells suggests that the cdc 22 gene product may be a negative element involved in histone H2A transcription.

2 Comparison of the cdc 22 Mutant with cdc Mutants Isolated in Other Organisms

The work of Jazwinski and Edelman (1976) showed that extracts from the budding yeast S. cerevisiae are capable of stimulating the initiation of DNA replication in frog cell nuclei. This suggested that many of the functions involved in the initiation of DNA replication have been conserved across a range of organisms. Recent results (Beach et al, 1982; Barker and Johnston, 1983) have shown that cloned CDC⁺ genes isolated from the budding yeast S. cerevisiae can complement defects in cdc mutants of S. pombe, which is considered a distantly related organism (Schweingruber and Dietrich, 1973). For example, the cloned cdc 9 gene of S. cerevisiae which codes for DNA ligase complements the S. pombe cdc 17 mutation, known to code for DNA ligase in this organism (Johnston and Nasmyth, 1978; Nasmyth, 1977; Barker and Johnston, 1983). A more unexpected observation was that the cloned cdc 28 gene of S. cerevisiae could complement a defect in the cdc 2 gene of S. pombe (Beach et al, 1982). Both these genes are known to be involved in their respective organisms in the major G₁ cell cycle control termed "start" (Reed, 1980; Nurse and Bisset, 1981). Once "start" is completed the cell becomes committed to the mitotic cycle rather than an alternative developmental pathway such as conjugation. Although the "start" control point divides the cell cycles of both yeasts into uncommitted and committed phases this does not necessarily mean that "start" has the same molecular mechanism in both organisms. The ability of the S. cerevisiae cdc 28⁺ gene to complement an S. pombe cdc 2 mutation provides strong evidence that the molecular basis of cell cycle control is highly conserved between the two organisms.

The above observations indicate that common mechanisms exist for controlling progress through the cell cycle in S. pombe and S. cerevisiae. Therefore it would be useful to compare the S. pombe cdc 22 mutation with S. cerevisiae cell cycle mutants that like the S. pombe cdc 22 mutation are required before the block imposed by hydroxyurea. In the next section the S. pombe cdc 22 mutation is compared first with the S. cerevisiae "start" mutants, and then with the cdc 4, dbf 4 and cdc 7 mutants which are required after "start" but before the initiation of DNA replication (Hereford and Hartwell, 1974; Johnston and Thomas, 1982).

(i) Comparison of cdc 22 with S. cerevisiae "start" Mutants

One particularly interesting group of genes identified in S. cerevisiae are those controlling "start". Cells with temperature sensitive mutations in the cdc 28, cdc 36, cdc 37, cdc 39 and others have been shown to be involved in the regulatory control point known as "start" (Reid and Hartwell, 1977; Reed, 1980). When incubated at the restrictive temperature haploid cells with mutations in "start" genes arrest in G_1 as unbudded cells.

Reid and Hartwell (1977) investigated the ability of different cdc arrested mutants to undergo conjugation with haploid cells of the opposite mating type. For these experiments conjugation was assayed by the formation of diploid cells. It was found that cells arrested at "start" were still able to conjugate while temperature sensitive cdc mutant strains that arrest after "start" were unable to conjugate. In this study the result obtained with blocked cdc 4 cells, which arrest after start (Hereford and Hartwell, 1974), could not be interpreted

as control experiments suggested that the cdc 4 gene product was required for the mating process. However, the conclusion from the above observations was that "start" defined the last point before commitment to the mitotic cell cycle.

This definition of "start" has been questioned in light of experiments carried out by Hirschenberg and Simchen (1977). Hirschenberg and Simchen (1977) tested the ability of diploid cells arrested at various cdc steps to undergo sporulation. They found that only diploid cells arrested at "start" or the cdc 4 steps were able to sporulate without completing an intervening mitotic cell cycle. This suggested that a cdc 4 diploid was not committed to the mitotic cell cycle since it was able to switch directly into the alternative developmental pathway of meiosis and sporulation. To reconcile these results, Nurse (1980) has proposed that "start" should be considered more as an area including the cdc 4 event rather than as a single control point in G_1 of the cell cycle.

In S. pombe two "start" genes have been identified, cdc 10 and cdc 2, by the ability to form diploid cells from their points of arrest. (Nurse and Bisset, 1981). A cdc 22 mutant strain when arrested was not able to form diploid cells and therefore would seem to be required after "start" had been completed. It is possible that the S. pombe cdc 22 mutation might define a point of commitment after "start" in an analagous manner to the S. cerevisiae cdc 4 mutant. However this seems unlikely as in contrast to the S. cerevisiae cdc 4 mutation the S. pombe cdc 22 gene product does not seem to be involved in the mating process.

(i) Comparison of *S. pombe* cdc 22 Mutant with the cdc 4, dbf 4 and cdc 7 Mutants of *S. cerevisiae*

In *S. cerevisiae* besides the "start" genes and cdc 4 the products of dbf 4 and cdc 7 are required during G_1 prior to the initiation of DNA synthesis (Johnston and Thomas, 1982; Hereford and Hartwell, 1974). Both the dbf 4 and cdc 7 mutants arrest with a similar phenotype of a dumbbell morphology (Johnston and Thomas, 1982). This phenotype differs from "start" mutants which arrest as unbudded cells and cdc 4 mutants which form elongated buds (Hartwell et al, 1973). By examination of the terminal phenotypes of double mutant cells it has been established that the "start" gene products are required before the cdc 4 gene product, which is in turn required before the products of dbf 4 and cdc 7.

A further step defined in the G_1 phase of the *S. cerevisiae* cell cycle is the point at which all the proteins necessary for a round of DNA synthesis have been synthesised. When cdc 7 and dbf 4 mutant cells are arrested at the restrictive temperature and the block released in the presence of cycloheximide, which blocks protein synthesis, DNA replication occurred in cdc 7 but not dbf 4 cells (Johnston and Thomas, 1982). This suggested that the dbf 4 gene function acted before the final requirement for protein synthesis which in turn was completed before the step defined by the cdc 7 gene product.

In *S. pombe* mutations in two genes, cdc 20 and cdc 22, have been shown to be defective in the initiation of DNA synthesis (Nasmyth and Nurse, 1981). It is unfortunate that the different terminal phenotypes associated with G_1 *S. cerevisiae* cdc mutants cannot be recognised in *S. pombe* cdc mutants because there is no morphological

marker early in the cycle comparable with budding in S. cerevisiae. However the time at which the cdc 20 and cdc 22 gene products function could be mapped relative to the final requirement of protein synthesis for DNA replication in S. pombe.

The availability of an efficient method of transformation and plasmid vectors that can be selected for in both types of yeast allows "cross complementation" experiments to be carried out with the cloned cdc genes from each organism. The cdc 4 and cdc 7 genes of S. cerevisiae have been isolated (Peterson et al, 1984; Heddele et al; 1984) and would be ideal genes with which to test for complementation of the S. pombe cdc 22 mutation. It would also be of particular interest to try to complement the S. cerevisiae cdc 4, dbf 4 and cdc 7 mutations using the cloned S. pombe cdc 22 gene and cdc 22 suppressor.

3 Periodic Patterns of Gene Expression During the Cell Cycle

As described in the Introduction most proteins appear to be synthesised continuously through the cell cycle. This section reviews the small number of proteins that seem to be cell cycle regulated. Where possible the level at which the regulation occurs is indicated.

(i) S. pombe

In the fission yeast S. pombe two enzymes show a periodic stepwise increase in activity through the cell cycle when measured in synchronous cultures. Both these enzymes, thymidine monophosphokinase (TMPK) and nucleoside diphosphokinase (NDPK), are involved in nucleotide metabolism. In both cases the level at which cell cycle regulation occurs, such as transcription or posttranscription, is unknown. In addition it has recently been shown that histone H2A mRNA in a cell peaks periodically at S phase although it is not known how the accumulation of histone protein is affected. (Aves et al, 1985; See Chapter 5; Figure 5.8).

(ii) S. cerevisiae

In the budding yeast S. cerevisiae a recent examination of approximately 900 proteins revealed 17 proteins that appeared to show periodic or modulated patterns of synthesis during the cell cycle. (Lorincz et al, 1982). Three of these proteins, synthesised exclusively in late G₁ and early S phase, were found to be the histones H2A, H2B and H4. This confirmed previous studies which showed that histones were synthesised periodically during the cell cycle. (Moll and Wintersberger, 1976; Hereford et al, 1981). Further studies in S. cerevisiae have recognised different elements involved in the cell

cycle regulation of histone gene expression. Experiments on synchronous cultures using the cloned histone H2A - H2B genes as hybridisation probes demonstrated that the level of histone H2A - H2B mRNA peaks periodically during S phase (Hereford et al, 1981). By pulse labelling synchronised yeast cells and hybridising the labelled RNA to cloned histone DNA immobilised on a filter it was established that the periodic appearance of histone mRNA was the result of increased transcription rate (Hereford et al, 1982). Additional experiments have shown that a second level of control also exists that operates post-transcriptionally by altering the stability of the histone mRNAs. (Osley and Hereford, 1981; Hereford et al, 1982).

The point in the cell cycle at which transcription is activated was defined more accurately using a cdc 7 temperature sensitive mutant. Cells with a cdc 7 mutation arrest in late G₁ before the initiation of DNA synthesis (Hereford and Hartwell, 1974). Cells blocked at the cdc 7 step start histone transcription even though DNA replication did not occur. This suggested the completion of an event in G₁ rather than the initiation of S phase switched on histone transcription. Moreover, in a cdc 7 blocked mutant the H2B transcription rate remained at a high level. This implied that a point later in the cell cycle was required to turn transcription off.

The point at which histone transcription was switched off was investigated by examining histone mRNA levels in a strain carrying a cdc 3 mutation. At the restrictive temperature cells containing a cdc 8 mutation will complete the cdc 7 gene function, initiate DNA synthesis and arrest early in S phase. In a blocked cdc 8 strain the histone transcription rate was found to peak in a similar pattern to wild type cells. The simplest explanation of this result is that

histone transcription switches off upon entry of cells into S phase.

Recently a DNA sequence required for transcriptional regulation has been identified at the 3' end of the H2B gene in each of the two genetically unlinked H2A -H2B copies (Osley and Hereford, 1982). An interesting observation is that the same sequence is also capable of supporting autonomous replication of plasmids otherwise unable to replicate autonomously in yeast. Autonomously replicating sequences (ars) have been proposed to be chromosomal origins of replication although they have not yet been shown to function as such in vivo (Struhl et al, 1979). Deletion mapping has shown that the ars activity and regulatory sequence required for histone transcription are coincident or within 100 bp of one another. Using this information Osley and Hereford (1982) proposed a simple method to explain how the periodic transcription of the H2A and H2B genes were temporally regulated. In this model changes in the chromatin structure brought on by activation of the eukaryotic origin of replication located at the 3' end of the H2A - H2B genes was responsible for the initiation of H2A - H2B gene transcription. When replication has taken place the chromatin structure returned to its normal state and histone transcription would cease. Thus the activation of the ars sequence once every cycle would be responsible for the periodic transcription of the H2A - H2B genes.

The enzyme thymidylate synthetase which is required for nucleotide precursor metabolism shows a peak in enzyme activity during S phase in synchronous cultures of S. cerevisiae. (Storms et al, 1984). The peak in thymidylate synthetase activity is paralleled by a peak in the amount of enzyme protein present in cell extracts. This implies that periodic synthesis of protein is responsible for the periodic peak in enzyme activity. Using the previously cloned thymidylate

synthetase gene (Taylor et al, 1982) as a hybridisation probe, the transcript level was found to be cell cycle regulated reaching a peak near the beginning of S phase. This indicates that the periodic increase and decrease in thymidylate synthetase protein levels is, at least partly, determined by the availability of thymidylate synthetase transcript.

The levels of transcript coding for DNA ligase in S. cerevisiae have recently been shown to peak periodically during late G₁ (Peterson et al, 1985). These authors presented no information about DNA ligase activity or enzyme protein levels at different cell cycle stages.

Another example of periodic gene expression in S. cerevisiae involves the homothallism HO gene. This gene codes or regulates an endonuclease essential for the G₁ specific event of mating type inter-conversion (Kostriken et al, 1983). The endonuclease activity first appears during early G₁ and thereafter increases for the rest of the cell cycle. Regulation seems to be at the transcriptional level as the accumulation of HO mRNA shows a similar pattern to endonuclease activity (Masmyth, 1983).

(iii) Physarum Polycephalum

Another well documented system is the acellular slime mould P. polycephalum. This organism shows a natural mitotic synchrony which avoids the perturbations that are inherent in other organisms using most methods of synchronisation. Mitchison (1977) states that out of fourteen enzymes assayed in Physarum, eight were continuous while six showed a periodic peak in activity during the cell cycle. Of the "periodic" enzymes, one histone H1 kinase was thought to be involved in chromosome condensation during mitosis and three other enzymes,

thymidine kinase, thymidylate synthetase and DNA polymerase, were associated with S phase. The peak in activity shown by histone H1 kinase is unusual because the observed increase in activity is not due to a parallel increase in the amount of enzyme synthesised (Mitchelson et al, 1978). This result implied that some form of post translational control was involved in the regulation of the periodic activity increase.

Experiments on Physarum involving pulse labelling of plasmodium proteins and subsequent separation by two dimensional gel electrophoresis showed that two polypeptides out of several hundred were preferentially synthesised during the late G₂ phase of the cell cycle. (Lafflèr et al, 1981). Further investigation identified one polypeptide as α tubulin and the other as β tubulin. α and β tubulin polypeptides polymerise to form microtubules which are the major macromolecular components of the mitotic spindle (Luduena, 1979).

A more detailed survey by Burland et al, (1983) found that two α tubulins (α_1 and α_2) and two β tubulins (β_1 and β_2) were expressed in the Physarum plasmodium. A recent report (Schedl et al, 1984), revealed that the synthesis of all four tubulin polypeptides is cell cycle regulated. The synthesis of all four tubulin species increased from early G₂ in a co-ordinate manner until metaphase, then fell rapidly. This increased rate of protein synthesis was paralleled by a corresponding increase in the steady state level of α and β tubulin mRNAs.

(iv) Animal Eggs

Unfertilised sea urchin or Xenopus eggs are naturally arrested in the metaphase of the second meiotic division. (Woodland, 1982;

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Newport and Kirschner, 1982). When stimulated by fertilisation the egg proceeds into a rapid and nearly synchronous series of cell divisions. Studies on these organisms have identified proteins with interesting periodic patterns of accumulation.

In the sea urchin Arbacia punctulata cleavage in embryos consists of eight very rapid divisions that require continual protein synthesis to maintain them. This synthesis is programmed by stored material mRNAs which code for three or four particularly abundant proteins whose synthesis is barely detectable in the unfertilised egg (Wells et al, 1981). The accumulation of one of these proteins, termed cyclin, has been shown to be periodic, peaking during late G₂. Pulse labelling experiments indicated that cyclin was synthesised continuously during the cell cycle. This observation indicated that periodic accumulation of cyclin may be due to periodic degradation of the protein (Evans et al, 1983).

Experiments performed with Xenopus eggs and oocytes revealed the presence of a factor, termed maturing promoting factor (MPF), responsible for the initiation of the meiotic or mitotic phase of the Xenopus cell cycle. MPF was first detected in unfertilised amphibian eggs as a cytoplasmic factor which when injected into amphibian oocytes caused them to "mature" into unfertilised eggs (Masui and Clarke, 1979). More recently MPF has been extracted in an active form from the cytoplasm of all eukaryotic mitotic cells investigated (Newport and Kirschner, 1984). MPF activity oscillates in the early cleavage stages of the Xenopus embryo, being highest at mitosis and undetectable at S phase (Gerhart et al, 1984). Of particular interest is the observation that MPF activity continues to oscillate in Xenopus embryo's when mitosis has been inhibited by the presence of nocodazole or

colchicine. This implies that the periodic fluctuations in MPF activity may be independent of the nuclear division cycle.

When comparing the observations obtained during cleavage in Xenopus and sea urchin fertilised eggs with the cell cycle regulation of gene expression in other organisms it is important to remember that in certain respects the cell cycle in fertilised eggs is atypical. For example, no net growth occurs between divisions; no transcription takes place during the cell cycle and the cycle does not contain a G_1 or G_2 phase (Newport and Kirschner, 1982). Therefore it is uncertain at present how these results relate to a normal mitotic cell cycle.

(v) Mammalian Cells

In mammalian cells the rate of histone protein synthesis has been shown to peak during S phase. The cell cycle regulation appears to be determined by the availability of histone transcript. The amount of histone transcript present seems to be controlled in a similar manner to that in S. cerevisiae with transcriptional and post-transcriptional control elements (Robbins and Borun, 1967; Plumb et al, 1983).

A great deal is known about the pattern of enzyme activities during the mammalian cell cycle. However due to problems in the methods of achieving synchronous cultures proper asynchronous control cultures cannot be obtained (discussed in detail in the Introduction). It is therefore unclear whether the observed periodicity in enzyme activity is a consequence of the method used to achieve synchronous cultures, or a property of the normal cell cycle.

(vi) Conclusion

In summary, each unambiguous example of periodic accumulation of protein and/or enzyme activity seems to be associated with a periodic event in the cell cycle such as DNA synthesis or mitosis. In most cases where it has been investigated the periodicity is, at least partly, due to regulation of gene transcription. This sometimes acted in conjunction with a post-transcriptional control involving the turnover of mRNA. However there are exceptions to this rule. For example regulation of histone kinase activity in Physarum appears to be the result of periodic activation of the enzyme. In addition regulation of periodic protein accumulation during cleavage in fertilised eggs during cleavage apparently involves post-transcriptional controls. This exception is not surprising as transcription does not take place during cleavage in fertilised eggs.

4 Cell Cycle Regulation of the cdc 22 mRNA

Using RNA obtained from a synchronous population of wild type cells prepared by elutriation it has been demonstrated that the cdc 22 transcript is cell cycle regulated (Chapter 5; Figure 5.8). A direct comparison of the cell number values corresponding to the different RNA samples showed that the cdc 22 transcript peaks approximately 15 minutes before the midpoint of the rise in cell number. This approximates to the transition from G₁ to S phases in wild type cells (Nasmyth et al, 1979). The peak in the level of histone H2A transcript also occurs at about this point in the cell cycle (Aves et al, 1985; see Chapter 5, Figure 5.9).

A number of S. pombe cdc genes have been isolated by using methods similar to those outlined in this thesis. The level of the cloned S. pombe cdc 2, cdc 10, cdc 17 and cdc 25 transcripts did not show periodicity through the cell cycle (Aves et al, 1985; Dr Paul Nurse and Dr L Johnston, Personal Communications). In contrast, the histone H2A transcript has been reported to show a dramatic fluctuation in its level during the cell cycle (Aves et al, 1985).

In this thesis evidence is presented that demonstrates that the level of the cdc 22 transcript is cell cycle regulated. The appearance of the cdc 22 transcript is restricted to a particular period of the cell cycle peaking at the boundary of G₁ and S phase. (Chapter 5, Figure 5.8). The cell cycle regulation of the level of cdc 22 mRNA could be explained by either periodic transcription of the cdc 22 gene, changes in the stability of the cdc 22 transcript at a particular point in the cell cycle or a combination of both. The observations reported in Chapter 5 cannot distinguish between either of the three possibilities. To test which mechanism is responsible for the periodicity in the level

of the cdc 22 transcript will require a detailed analysis of the cdc 22 transcription rate through the cell cycle. This could be carried out by pulse labelling a synchronous culture with a radioactive nucleotide, extracting total RNA and hybridising to S. pombe DNA containing only the cdc 22 sequence. Such a procedure was used to clearly establish that both periodic transcription and changes in mRNA stability were responsible for the cell cycle regulation of histone mRNA in S. cerevisiae (Hereford et al, 1982).

The cdc 22 transcript has been shown to be present at high levels during the G₁/S boundary and basal levels at other times in the cell cycle. If the periodicity in the level of the cdc 22 transcript was dependent on cell cycle progress, arresting cells in late G₁ or S would result in high levels of cdc 22 mRNA being present. In contrast, the cdc 22 transcript would be present in a low amount in cells blocked G₂ or mitosis. Experiments described in Chapter 5 demonstrate that the cdc 22 transcript is present at the same abundance as an unblocked wild type control in RNA obtained from blocked asynchronous cultures of six different mutant strains. Four of the mutants cdc 10, cdc 20, cdc 22 and cdc 17 arrest at late G₁ or S phases while two, cdc 2 and cdc 1, arrest during late G₂. The interpretation of this result is that the appearance of the cdc 22 transcript is not dependent on the completion of any specific cell cycle event. This leads to the intriguing possibility that the periodicity in the cdc 22 transcript level may be independent of continued progress through the cell cycle. Such a type of regulation is not without precedent. For example, cell cycle regulation of MPF activity during cleavage in Xenopus embryos, discussed in the last section, is thought to show a similar pattern (Gerhart et al, 1984). In addition, when the cdc 4 mutant of S. cere-

visiae is incubated at the restrictive temperature bud formation occurs repeatedly after constant time intervals (Hartwell et al, 1974).

Direct support for the hypothesis that the periodicity in the cdc 22 transcript level is independent of the nuclear division cycle would involve an investigation of the cdc 22 transcript levels in blocked synchronous cultures of various S. pombe cdc mutants.

The regulatory sequence responsible for periodic expression of histone H2A - H2B in S. cerevisiae is coincident with a DNA sequence that supports autonomous replication (ars) of plasmids in yeast (Osley and Wereford, 1982). Such ars sequences are also thought to be involved in the regulatory regions required for the DNA ligase and HO endonuclease periodicities (Peterson et al, 1985; Nasmyth, 1983). No such ars activity was detected in the cloned S. pombe DNA carrying the cdc 22⁺ gene (Chapter 4). However such an observation does not rule out the possibility that an ars DNA sequence could be responsible for the cdc 22 transcript periodicity as it is not known if all the regulatory sequences responsible for the cell cycle fluctuations are present on the cloned S. pombe DNA sequence.

5 cdc Suppressor Sequence(i) Properties of the Suppressor Sequence

A 2.2 Kb S. pombe DNA sequence is described in Chapter 6 that complements both the known mutant alleles of the cdc 22 gene. When this sequence is integrated by homologous recombination into the S. pombe genome the site of the integration is genetically unlinked to the cdc 22 locus. Thus, the 2.2 Kb sequence contains an extragenic suppressor of cdc 22 mutations. Suppression only occurs when the 2.2 Kb DNA sequence is present on an autonomously replicating vector. When integrated into the S. pombe genome it no longer suppresses the cdc 22 - 145 or cdc 22 - C11 mutations. This suggests for suppression to occur requires the DNA suppressor sequence to be present at an elevated copy number.

Suppression of mutations by cloned extragenic DNA fragments carried on autonomously replicating plasmids has been reported by a number of workers. MacKay (1983) clearly demonstrated that the cloned STE 5 gene of S. cerevisiae also suppresses a ste 4 mutation when carried on an ars plasmid. Another example of such suppression in S. cerevisiae is that the cloned CDC 12 gene will also suppress a cdc 11 mutation (Lillie et al, 1984).

In S. pombe a DNA sequence has been isolated from a plasmid library that suppresses certain cdc mutant alleles. This sequence, termed suc 2, was found to be unlinked to the cdc 2 locus (Hayles and Nurse, 1984).

Theoretically, a number of different mechanisms can be put forward to explain suppression of the cdc 22 mutation by an extragenic DNA sequence carried on a plasmid. Some of these are considered in the following section.

Possible Methods of Obtaining Suppression

A clue to the mechanism of suppression is the observation that suppression only occurs when the sequence is present on a plasmid capable of autonomous replication in S. pombe (See Chapter 4). When the DNA sequence responsible for suppression was integrated into the S. pombe genome no complementation of cdc 22 - M45 or cdc 22 - C11 mutant cells occurred. From the autoradiograph shown in Chapter 4, Figure 4.8, it was demonstrated that a single plasmid containing the S. pombe suppressor sequence had integrated into the S. pombe genome. The plasmid vector pDE243 in S. pombe is reported to be present at a copy number of five per yeast cell (Wright et al, 1985). This suggests that suppression of the cdc 22 mutation is only successful when greater than two copies of the cdc 22 suppressor DNA is present. The simplest explanation for this increased copy number requirement is that the suppressor gene product must be overproduced for suppression of the cdc 22 mutation to occur. Allowing for the possibility that the suppressor gene product is required to be overproduced a number of different models can be proposed to explain the observed suppression of the cdc phenotype.

Two possible mechanisms require that the cdc 22 mutant gene product is partially active at the restrictive temperature. In the first the suppressor gene product catalyses the reaction before the cdc 22 gene product in the same biochemical pathway. An increased amount of suppressor product would increase the substrate concentration for the reaction catalysed by the cdc 22 gene product. This could drive the reaction, compensating for the partially defective cdc 22 gene product. Alternatively the suppressor gene product could regulate the expression of the cdc 22 gene. Increasing the amount of suppressor

gene product would also increase the amount of cdc 22 gene product produced. Although this cdc 22 mutant gene product is partially active, increasing the amount produced would partially compensate for the defect. For both these mechanisms the suppression would be expected to be allele specific.

Another possible explanation could be that the suppressor DNA sequence coded for a protein of a similar biochemical function to the cdc 22 gene product. For example, both could code for protein kinases of different specificities. Though the suppressor gene product would not be expected to function as efficiently as the cdc 22 gene product, it could **bypass** the biochemical defect provided enough of the suppressor protein was available. Such a mechanism would be expected to be a gene rather than allele specific.

An alternative mechanism could involve stabilising the mutant protein at the restrictive temperature. This could be achieved by the suppressor protein interacting directly with the cdc 22 gene product. Alternatively the suppressor gene product might act to stabilise the mutant protein by changing the internal environment of the cell in a general way. For example, it has already been observed that the cdc 22 - M45 strain is osmotically suppressible if grown in the presence of 1.2 M sorbitol at the restrictive temperature. (See Chapter 3). Osmotic suppression is thought to act by stabilising the mutant protein in response to a reduction in the concentration of water surrounding the molecule (Fincham et al, 1979). Suppression mechanisms which act by stabilising a mutant protein would be expected to be allele specific.

The cdc 22 suppressor sequence suppresses both known mutant alleles of the cdc 22 gene. As both the cdc 22 alleles have different genetic and physiological properties it seems likely that a gene rather than

allele specific mechanism is responsible for suppression. Of the different methods described, only the bypass mechanism would be expected to be gene rather than allele specific. Therefore this suggests that suppression of the cdc 22 mutations may occur by a bypass mechanism.

Transcriptional Analysis Using the Cloned Suppressor Sequence

When total RNA from wild type cells grown at 25°C was probed with the 2.2 Kb DNA fragment by Northern analysis a single transcript of 1.5 Kb in size was detected (Chapter 6, Figure 6.1). However, when RNA isolated from six different blocked cdc mutant strains was hybridised to the 2.2 Kb DNA fragment a surprising pattern of transcript levels was obtained. In RNA obtained from cdc 1, cdc 10, cdc 17, cdc 20 and cdc 22 blocked mutant strains a second transcript of 2.4 Kb in size was detected in addition to the 1.5 Kb transcript. The 2.4 Kb transcript was present at approximately half the abundance of the 1.5 Kb transcript. Curiously the larger transcript was present at a much reduced level in a blocked cdc 2 strain and wild type cells incubated at the same temperature (Chapter 6, Figure 6.3). Therefore, shifting certain cdc mutant strains to the restrictive temperature increases the level of the 2.4 Kb transcript. As wild type cells shifted to the restrictive temperature did not show this response it was thought unlikely that the increase in temperature led to the higher abundance of the 2.4 Kb transcript. A more plausible explanation was that cell cycle arrest accounted for the increased 2.4 Kb transcript level.

The level of the transcripts were also investigated through the cell cycle in RNA obtained from a synchronous culture of wild type cells

grown at 35°C. (Chapter 6, Figure 6-5). Only the 1.5 Kb transcript was easily detected. In contrast to the cdc 22 mRNA no **cell cycle regulation** in the level of the 1.5 Kb transcript was observed through the cell cycle.

Relationship of the Two Homologous Transcripts to the Cloned DNA Fragment

An important unresolved question is to establish the relationship between both the 1.5 Kb and the 2.4 Kb transcripts to the cloned cdc 22 suppressor fragment DNA. At least three possibilities exist.

First, the two transcripts could be coded by the same gene. For example, if the suppressor gene contained one or more introns, the smaller transcript could represent the final spliced form of the larger transcript. An alternative explanation could be that both transcripts are coded by the same gene but are transcribed with different 5' and, or 3' ends. Such a mechanism has been described for transcription of the SUC 2 gene of S. cerevisiae (Carlson and Botstein, 1982). The SUC 2 gene codes for two different forms of invertase, an intracellular and a secreted form. The two forms differ at the 5' end of the mRNAs.

A second possibility is that the transcripts are from different genes located next to each other in the S. pombe genome.

An unlikely, though still possible explanation is that the two different transcripts are transcribed from opposite strands of the same DNA fragment.

To find out which of the above possibilities is an accurate reflection of the relationship between the two transcripts, will involve further investigation. For example, S₁ mapping (Berk and

Sharp, 1977) would provide evidence for the presence or absence of introns. In S_1 mapping the hybridisation conditions that favour the formation of DNA-RNA hybrids are provided. This allows a DNA fragment to hybridise to the mRNA encoded by it. Any introns in the DNA coding sequence will form single stranded loops. These single stranded loops are removed by the enzyme, S_1 nuclease which specifically digests single stranded DNA. The product of this reaction is a DNA-RNA hybrid, the length of the mRNA. Any introns will result in breaks in the DNA strand. Treatment with alkali removes the RNA, releasing the individual fragments of DNA. These identify the individual exons of the gene.

Alternatively, the possibility that the two transcripts were transcribed from opposite strands of the same DNA sequence could be investigated using single stranded DNA as hybridisation probes. Single stranded DNA probes are easily obtained by cloning a double stranded DNA fragment into a vector based on the M13 bacteriophage (Messing, 1983).

Which Homologous Transcript is Responsible for Suppression?

The suppressor DNA was isolated by the ability to rescue cdc 22 mutations at the restrictive temperature of 36°C . At this temperature both the 2.4 Kb and 1.5 Kb transcripts were easily detected in a blocked cdc 22 - M45 strain (Chapter 6, Figure 6.3). Therefore either of the two transcripts could code for the gene product responsible for suppression of cdc 22 mutations. To demonstrate which transcript is necessary for suppression would involve subcloning the 2.2 Kb DNA sequence into defined fragments. Each subclone would then be tested in vivo for suppression of a cdc 22 mutant using yeast transformation

experiments. The information gained on the relationship of the 2.4 Kb and 1.5 Kb transcript to the 2.2 Kb DNA sequence would be beneficial in deciding the experimental strategy to be used in the subcloning experiments.

Possible Function of the Suppressor Fragment?

Although it is known that the cdc 22 suppressor fragment is able to rescue cdc 22 mutations it would be interesting to establish if this suppressor gene is essential for cell viability. One way of testing this possibility would be to disrupt the gene using the method of Rothstein (1983). This method takes advantage of previously reported observations that during yeast transformations free DNA ends are recombinogenic, stimulating the recombination by interacting with homologous DNA sequences in the yeast genome (Orr-Weaver et al, 1981). The principle of the method is shown in Figure 9.1. The cloned DNA fragment containing the gene Y to be tested is digested with a restriction enzyme that cuts within the gene Y sequence. A selectable S. pombe gene such as Ura 4⁺ is inserted into gene Y disrupting it. The in vitro disrupted gene is then released from the plasmid sequences by restriction enzyme digestion and used to transform a ura 4⁻ gene Y⁺ yeast cell and URA 4⁺ transformants selected. In these cells the gene Y⁺ has been replaced with a disrupted gene Y. The method can be used to determine whether a gene is essential by disrupting the gene in a "partial diploid". In a "partial diploid", the haploid cell contains the gene of interest carried on an autonomously replicating plasmid. If the disrupted gene is essential, loss of the plasmid should be lethal to the haploid yeast cell.

Figure 9.1

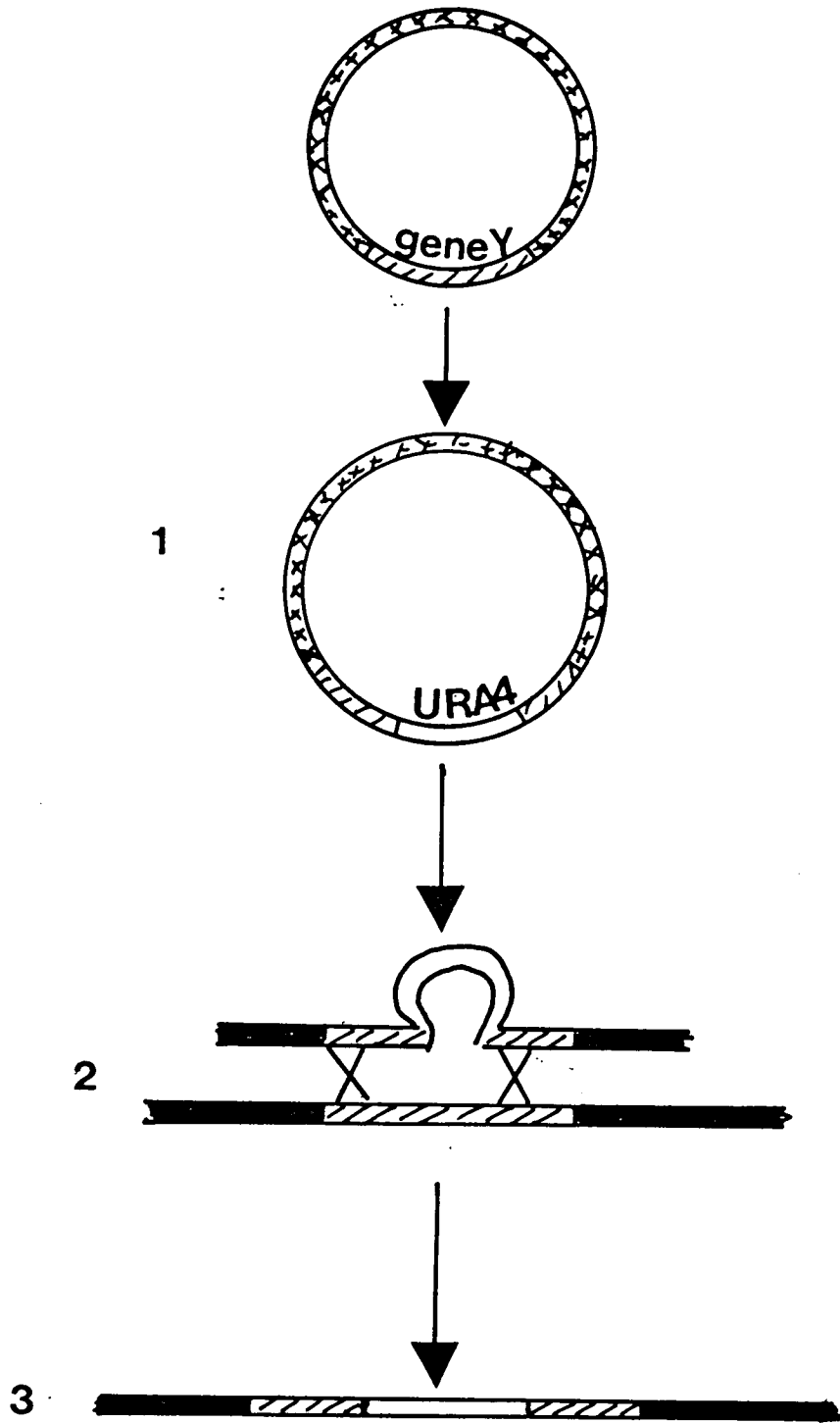





Figure 9.1 Principle of Gene Disruption

- 1 Subclone URA^+ into gene Y DNA sequence, disrupting it.
- 2 Cut either side of URA^+ gene, in the yeast DNA sequence. Transform a $ura4^-$ gene Y^+ yeast.
- 3 Select URA^+ transformants. In these cells the gene Y^+ has been replaced by the disrupted gene Y.

 Plasmid vector DNA

 Gene Y

 Ura 4

 S. pombe chromosomal DNA

If disruption of the cdc 22 suppressor gene was lethal to S. pombe cells a conditional lethal mutant could be obtained by in vitro mutagenesis. Shortle et al (1984) recently constructed temperature sensitive mutations of the S. cerevisiae actin gene by in vitro mutagenesis of the cloned gene. Of particular interest would be the possibility of a conditional lethal mutant of the cdc 22 suppressor conferring a cdc phenotype.

Possible Mechanisms for the Elevated Level of the 2.4 Kb Transcript in Certain Blocked Asynchronous cdc Cells

In the **experiments** described in Chapter 6 it was demonstrated that two different transcripts were homologous to the cdc 22 suppressor DNA. One transcript was 1.5 Kb in size and was always present at approximately the same abundance in RNA prepared from wild type or any of six different blocked cdc mutant strains. The other transcript was 2.4 Kb in size and was present at a low level in RNA obtained from wild type cells and a cdc 2 blocked mutant strain. In contrast this 2.4 Kb transcript was found at an elevated level in RNA prepared from a cdc 1, cdc 10, cdc 17, cdc 20 and cdc 22 blocked mutant cells.

At least two possibilities could account for this increased level of the 2.4 Kb transcript in certain blocked cdc strains. The first is that the increased level is obtained as a result of imposing a block in the cell division cycle. If this was the case it would suggest that the cdc 2 gene product was itself involved in the regulation of the level of the 2.4 Kb transcript. This is because out of six cdc mutant strains tested a low level of the 2.4 Kb transcript was only found in RNA obtained from the blocked cdc 2 strain. As wild type cells are not blocked in cell cycle progress they do not exhibit an elevated level of the 2.4 Kb transcript.

An alternative explanation could be that the increased level of the 2.4 Kb transcript was observed as a result of blocking a particular cell cycle event. Four of the cdc mutants that show the elevated level in the 2.4 Kb transcript cdc 10, cdc 17, cdc 20 and cdc 22 arrest at or near S phase (Nurse et al, 1976; Nasmyth and Nurse, 1981). Therefore, a possible mechanism could be that arrest at S phase could induce the elevated level of the 2.4 Kb transcript in an analogous manner to the "SOS" phenomenon in E. coli (Little and Mount, 1982). The cdc 2 gene product is required at two points in the cell cycle, at G₁ and late G₂ (Nurse and Bisset, 1981). However in a blocked cdc 2 asynchronous culture greater than 90% of the cells will be arrested at the G₂ block, due to the short G₁ associated with the S. pombe cell cycle (Nasmyth et al, 1979). Therefore, a possible explanation of the reduced level of the 2.4 Kb transcript in a cdc 2 blocked culture could be due to most cells arresting at late G₂. However, for this explanation to be correct it must be assured that the cdc 1 blocked cells, which arrest after the cdc 2 block in late G₂ (Fantès, 1982), is atypical compared to other cdc mutants that arrest at mitosis.

To determine which, if any, of the two possibilities is correct would require the investigation of a number of additional cdc mutants that block at either S phase or mitosis. It would also be of interest to determine if cell cycle arrest by the use of chemical inhibitors such as hydroxyurea, which arrests cells at S phase (Mitchison and Creanor, 1971), or MBC, which blocks cells at mitosis (Fantès, 1982), also caused an increase in the level of the 2.4 Kb transcript.

A number of treatments such as irradiation with UV or exposure to

a number of DNA damaging chemicals are known to induce the "SOS" response in bacteria (Little and Mount, 1982). If such treatments also caused elevated levels of the 2.4 Kb transcript this could provide evidence that DNA damage was required to produce the response.

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ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr Peter Fantes for his constant advice and encouragement during the course of this project. I also appreciate him not moaning (well not much!) when he found that most of his glassware and solutions had 'mysteriously' disappeared.

A thank you must also go to all the members of the Edinburgh S. pombe cell cycle group. In particular, Dr Jim Creanor for his advice on the preparation of synchronous cultures and Professor Murdoch Mitchison for stimulating discussions on all aspects of the cell cycle. I would like to thank Dr Douglas Roy for demonstrating most of the basic skills for working with S. pombe. I would like to express my thanks to David Hughes and Dr Douglas Roy for many interesting conversations both inside and outside working hours on many facets of cell cycle biology. A special mention must go to Joan Davidson who managed (somehow) to clean all the debris remaining from my experiments.

Thanks are also due to all members of the Zoology Department at Edinburgh University, particularly on the 3rd and 4th floors, who made the department such a pleasant place to work.

Finally, I would like to thank the members of my family for their constant support (and food parcels) during the period of this work which was greatly appreciated. Last but not least I would like to express my appreciation to Joyce Gordon who had the unenviable task of typing this thesis which she completed so expertly under almost insurmountable odds (my handwriting).