Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2

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A human homologue of the cdc2 gene has been cloned by expressing a human cDNA library in fission yeast and selecting for clones that can complement a mutant of cdc2. The predicted protein sequence of the human homologue is very similar to that of the yeast cdc2 gene. These data indicate that elements of the mechanism by which the cell cycle is controlled are likely to be conserved between yeast and humans.

The cdc2 gene plays an important role in controlling the cell cycle of the fission yeast Schizosaccharomyces pombe\(^1\). In this article we describe the isolation and characterization of a cdc2 homologue from human cells. The gene was cloned by expressing a human cDNA library in S. pombe and selecting those clones which could complement a mutation in the cdc2 gene. This method is applicable for isolating other mammalian genes for which mutants in equivalent genes are available in yeast. The human CDC2 gene has been sequenced, and potentially encodes a protein of the same molecular weight as S. pombe cdc2 with a 63% identity of amino-acid residues. This functional and structural similarity indicates that elements of cell cycle control are likely to be conserved between yeast and humans, and therefore will probably be found in all eukaryotic cells.

Cell cycle control

The cdc2 gene encodes a function which is required at the two major control points during the S. pombe mitotic cell cycle\(^2\). The first control is called start and is located in G1 at the point where the cell becomes committed to the mitotic cell cycle. Once start is passed, the cell cannot undergo the alternative developmental fate of conjugation until the cycle is reinitiated, and the cell also begins the programme of events which lead to S-phase\(^3\). The second control is located in G2 and determines when the cell initiates mitosis,\(^4\)\(^-\)\(^6\). Recessive temperature sensitive lethal cdc2 mutants which lack cdc2 function are unable to proceed past either the G1 or G2 control points. Dominant cdc2 mutants which contain a cdc2 function with altered regulatory properties traverse G2 more rapidly and initiate mitosis and cell division at a reduced cell size compared with wild type cells\(^7\). The cdc2 gene has been shown to encode a phosphoprotein of relative molecular mass 34,000 (M, 34K) which has protein kinase activity\(^8\). In nutrient-deprived cells the protein is dephosphorylated and has no kinase activity. On readdition of nutrients the protein gains kinase activity and becomes phosphorylated. This suggests that entry into the mitotic cell cycle could be regulated by modulating cdc2 protein kinase activity, possibly by phosphorylation\(^9\). Three genes are important in controlling the cdc2 gene function at the initiation of mitosis; cdc25\(^10\)\(^-\)\(^\)\(^11\) and nii1\(^12\) act as activators and wee1\(^13\) acts as an inhibitor. A fourth gene suc1 probably also interacts with cdc2 gene function\(^14\)\(^-\)\(^15\).

A gene analogous to cdc2 is found in the evolutionarily divergent budding yeast, Saccharomyces cerevisiae, and is called CDC28\(^16\). This gene function is required at start\(^17\) and probably later in the cycle for mitosis\(^18\). It encodes a 36K phosphoprotein with protein kinase activity\(^19\), and has 62% identity in protein sequence to the cdc2 gene product\(^20\). The similarities in structure and function of the cdc2 and CDC28 genes indicate that aspects of cell cycle control are conserved in the two yeasts.

Strategy for cloning

Three strategies can be employed to clone human homologues of known yeast genes: 1, low stringency hybridization to detect shared nucleotide sequences; 2, antibody screening of expression libraries to detect shared structural features; 3, complementation of mutants to detect genes with similar functions. We have used the third of these strategies, employing a human cDNA library to select for a gene that can complement a defective cdc2 function. This approach enables genes to be isolated not because of their structural similarities but because they can provide a similar function\(^21\)\(^-\)\(^23\). A human cDNA library was used because all mammalian introns cannot be expected to be spliced out in S. pombe. Because plasmids do not require an autonomously replicating sequence (ARS) for S. pombe transformation\(^23\) and the SV40 early promoter works well in S. pombe, we were able to use the excellent full-length Okayama and Berg human cDNA library made in an SV40 expression vector\(^24\). This library was co-transformed with another vector containing a selectable marker allowing cells that had taken up plasmid to be tested for their ability to grow in the absence of the cdc2 gene function. The vector used was pDB262 which transforms S. pombe very efficiently and contains the S. cerevisiae LEU2 gene able to complement S. pombe leu1–32 (ref. 25).

Cloning CDC2Hs

The S. pombe temperature sensitive mutant strain cdc2–33 leu1–32 was co-transformed with the plasmid vector pDB262 and the human cDNA library in the Okayama and Berg expression vector. A total of 10\(^7\) leucine prototrophic transformants were grown for 24 h at the permissive temperature of 29 °C and were then shifted to the restrictive temperature of 36 °C. Five transformants were found to form colonies. All of these were unstable for growth at 36 °C, indicating that the complementing activity was located on a plasmid. Two to four library plasmids were recovered into E. coli from each of the original five transformants. Each of these was tested for the ability to complement cdc2–33 on retransformation with pDB262 back into the cdc2–33 leu1–32 S. pombe strain. Two plasmids, pOB231 and pOB245, isolated from two different original yeast transformants, were found to complement the cdc2–33 mutation. Restriction mapping demonstrated that the plasmids were identical, each containing a similar 2-kilobase (kb) insert. A restriction map of this is shown in Fig. 1a. BamH I sites are located just outside the GC and AT tails in the vector\(^26\), and thus a BamH I digest of pOB231/245 removes the cDNA insert together with a small amount of flanking vector sequence. To establish if the other three original yeast transformants contained sequences related to pOB231/245, a Southern blot was made of BamH I digested DNA prepared from the five yeast strains and was probed with.
the insert prepared from pOB231. Hybridizing sequences were detected in all five transformants (Fig. 1b, tracks 1, 2, 4, 5, 6) whereas no hybridization was seen in an untransformed strain (Fig. 1b, track 3). Considerable plasmid rearrangement appears to have taken place in all five yeast strains. Only the two yeast transformants which yielded pOB231 and 245 (Fig. 1b, tracks 4, 5) contained a band corresponding in size to the 2-2 kb BamHI fragment from pOB231 found in pOB231/245.

These experiments establish that all of the original yeast transformants contained sequences related to the insert of pOB231 and that this plasmid could complement the cdc2-33 mutation. Our failure to recover complementary plasmids from the three other yeast transformants was probably due to plasmid rearrangements occurring in yeast which prevented their recovery in E. coli.

To prove that the insert in pOB231 could provide all the functions encoded by cdc2, the 2-2 kb BamHI insert was transferred to the LEU2 and 2-2 μm containing S. pombe plasmid vector pSB1 (pDB248X25 with a Sall site deleted) to generate plasmid pSB2H. This plasmid complemented all five cdc2a mutants tested, cdc2-36, L7, M26, M55 and M63, and also enabled an S. pombe strain deleted for cdc2 to grow normally. The deletion strain was constructed as shown in Fig. 1c. Initially a diploid strain was made which contained cdc2-36 on one chromosome homologue, and a deletion of the entire cdc2 open reading frame with an insertion of the marker ura4* on the other homologue. After transformation with pSB2H the diploid was sporulated. Those spores which contained the chromosome deleted for cdc2 germinated but failed to undergo cell division, forming elongated cells. But if the spores contained pSB2H, cells lacking cdc2* could divide like wild-type cells and formed colonies. Because the cells lose the plasmid
Fig. 2  a, Southern blots of DNA prepared from a wild-type strain and the cdc2Sp deletion strain containing pSAB23Hs. Lane 1, deletion strain probe with cdc2 (25p); lane 2, wild type probe with 2Sp; lane 3, deletion strain probe with pOB231 DNA insert (2Hs); lane 4, wild type probe with 2Hs. BamHI digestion generates a 7-kb fragment containing the cdc2Sp gene and a 2-kb fragment containing the pOB231 insert. b, Southern blots of DNA prepared from S. pombe, H. sapiens and C. cerevisiae probe with pOB231 insert (2Hs). Lane 1, S. pombe DNA; lane 2, H. sapiens DNA; lane 3, S. cerevisiae DNA. No significant hybridization is seen in S. pombe or C. cerevisiae, whereas a strong band of 8 kb is seen in H. sapiens. c, Northern blots of RNA prepared from S. pombe wild-type cells and H. sapiens cell line HT29. Lane 1, S. pombe RNA (Sp) probe with cdc2Sp (25p); lane 2, H. sapiens RNA (Hs) probe with 2Sp; lanes 3, 5, S. pombe RNA (Sp) probe with pOB231 DNA insert (2Hs); lanes 4, 6, H. sapiens RNA (Hs) probe with 2Hs. Lanes 5, 6 are longer exposures of lanes 3, 4.

Methods. Polyadenylated RNA was prepared from S. pombe wild-type cells as described and probed with purified fragments of the pOB231 cDNA insert and cdc2Sp made radioactive by oligolabelling using hexadecynylated primers.

frequently these colonies constantly produce highly elongated cells which divide no further (Fig. 1d). To establish that cells from these colonies had all their cdc2 sequences deleted and contained the pOB231 insert, DNA was prepared and compared with DNA from wild-type cells on a Southern blot. Probing with the pOB231 insert showed hybridizing sequences in the growing deletion clone but not in wild-type (Fig. 2a, tracks 3, 4), whilst probing with cdc2 showed the gene in wild-type but not in the growing deletion strain (Fig. 2a, tracks 1, 2). Therefore the pOB231 human cDNA insert can provide all the functions of S. pombe cdc2. We shall call the gene from which this cDNA is derived the human CDC2 gene. When necessary to avoid ambiguity we will use the suffix Hs (CDC2Hs) to indicate its Homo sapiens origin and Sp (cdc2Sp) when referring to the S. pombe cdc2 gene.

Human origin of CDC2Hs

It was important to demonstrate that CDC2Hs was of human origin. To do this DNA was prepared from H. sapiens, S. pombe and S. cerevisiae and digested with BamHI. S. cerevisiae was also included in this experiment as it contains the gene CDC28 which can complement mutants of S. pombe cdc2. After preparing Southern blots the DNAs were probed with CDC2Hs. No significant hybridization was observed with S. pombe or S. cerevisiae, but a major band of 8 kb and a minor band of 6 kb were observed with H. sapiens (Fig. 2b). From the band intensity the gene does not appear to be dispersed throughout the human genome and is likely to be single or low copy. To establish whether the gene is expressed in human polyadenylated RNA was prepared from the human colon carcinoma cell line HT29, together with S. pombe polyadenylated RNA. Both RNAs were Northern blotted and probed with cdc2Sp and CDC2Hs. A 1.8-kb transcript hybridizing to cdc2Sp was seen in S. pombe but not in H. sapiens (Fig. 2c, tracks 1, 2), whereas a 2.0-kb transcript hybridizing to CDC2Hs was seen in H. sapiens (Fig. 2c, tracks 4, 6) but not in S. pombe, even after over-exposure of the autoradiograph (Fig. 2c, tracks 3, 5). This confirms that CDC2Hs is of human origin and is not derived from S. pombe or S. cerevisiae, and that it is derived from a 2-kb polyadenylated transcript. As this is the length of the cDNA clone isolated it can be assumed that this clone is full length or almost so.

Sequence homology of CDC2Hs

The 2-kb BamHI fragment containing CDC2Hs was sequenced by subcloning into pTZ18 and pTZ19 (Pharmacia Ltd., Molecular Biology Division) using exonuclease III deletion and Alul and Haelll digestion. An extensive open reading frame (ORF) was located in the 1-kb fragment passing through the KpnI and BglIII sites. Sequencing both strands in this region confirmed that CDC2Hs potentially codes for a protein of 297 amino acids. The nucleotide sequence and its translation are given in Fig. 3.

The predicted ORF shares extensive homology with the fission yeast gene cdc2Sp and the budding yeast gene CDC28 (Fig. 4). Amino-acid identities throughout the proteins are 63% between CDC2Hs and cdc2Sp and 58% between CDC2Hs and CDC28. If only those regions which are identical between cdc2Sp and CDC28 are compared with CDC2Hs the identity rises to around 80%. The overall length of the three proteins are almost identical at 297 amino acids for CDC2Hs, cdc2Sp and 298 amino acids for CDC28. The predicted ORF of CDC2Hs also contains the two consensus regions surrounding the ATP binding (Fig. 3, nucleotides 170-229) and phosphorylation sites (Fig. 3, nucleotides 519-728) found in protein kinases suggesting that it encodes a protein kinase. Despite the conservation at the protein level, there are no stretches longer than 14 nucleotides which are the same between CDC2Hs and cdc2Sp.

The protein sequences of CDC2Hs and cdc2Sp are remarkably similar. Comparisons of histone H2A/B and a/b tubulin proteins, which are very closely related proteins, show identities between S. pombe and mammalian cells of between 68%-76% 27-29, not much greater than the 63% we have observed here. We propose that CDC2Hs encodes a functional and structural homologue of the fission yeast cdc2Sp gene. The human gene CDC2Hs is also likely to be the functional homologue of the budding yeast CDC28 gene, given its structural similarity to this gene.

A cdc2Sp like gene has been isolated from a human HeLa cDNA library using synthetic oligonucleotide probes corresponding to regions of expected similarity based on the sequences of conserved stretches within the catalytic domain of serine kinases. This cDNA has been partially sequenced and shows 47% identity in protein sequence to cdc2Sp. But it is different from CDC2Hs and its overall similarity to cdc2Sp is less than that found for CDC2Hs.

Human protein of M, 34K

To investigate the CDC2Hs gene product in human cells, antibodies were raised against a peptide represented by the single letter amino-acid code EGVPSTAIRELKE, which
Fig. 3 DNA sequence and its translation of pOB231 insert. Some 6-base recognition restriction enzyme sites are shown above the sequence. The ORF from the first initiator codon to the stop codon is given.

corresponds to nucleotide positions 264-311 (Fig. 3) and is not conserved in other protein kinases (Fig. 3). The serum detected a protein of 34K relative molecular mass on Western blots of protein prepared from the human colonic carcinoma cell line HT29 (Fig. 5a, track 1; Fig. 5b, track 1). A similar-sized protein was also detected in S. pombe cells (Fig. 5b, track 2 and Fig. 5c, track 1). This protein was shown to be the cdc2Sp gene product by demonstrating that in a yeast strain overproducing cdc2Sp transcript the 34K band is increased in intensity (Fig. 5b, track 3). The serum was also shown to detect the CDC2Hs protein by Western blot analysis of protein made from the yeast strain deleted for cdc2Sp but containing pSAB2Hs. This strain contained no cdc2Sp sequences and so could not contain any cdc2Sp protein. A 34K protein of similar size to that seen in human cells was detected in this strain (Fig. 5c, track 3; Fig. 5d, track 1). Cells deleted for cdc2Sp and not containing pSAB2Hs generated by germinating spores harbouring the chromosome with the deletion, had no 34K protein detectable by the serum (Fig. 5c, track 2). The anti-peptide serum detected a similar-sized protein in three other human cell lines, J6 T cells, HeLa HeLa-derived cells and Daudi B cells (Fig. 5d, tracks 2-4). A second serum raised against the carboxy-terminal 99 amino-acid residues of cdc2Sp (provided by V. Simanis) also detected a 34K protein in these three cell lines (Fig. 5e, tracks 1-3). We have also detected a 34K protein in untransformed Butler human embryo fibroblast cells using the anti-peptide serum (data not shown). These data suggest that the 34K protein is likely to be the CDC2Hs gene product.

The level of 34K protein produced in the cdc2Sp deletion strain containing pSAB2Hs (Fig. 5c, track 3) is similar in level to that produced by cdc2Sp in wild-type cells (Fig. 5c, track 1). This suggests that complementation of the fission yeast cdc2Sp gene function can be achieved without overproducing the human CDC2Hs protein.

Human genes in yeast

The experiments we have described provide an alternative approach for isolating homologous genes to using reduced stringency DNA hybridization or antibodies with a bacterial expression library. For the complementation approach to be successful the cDNA probably must be full-length and should be made in a vector that can transform and express the cDNA in yeast. The Okayama and Berg library met these criteria in fission yeast but probably new vectors would have to be developed if other simple eukaryotes such as S. cerevisiae or Aspergillus nidulans were to be used instead.

In addition to allowing the isolation of human genes by complementation, S. pombe can be used as an experimental system for investigating human gene function. The power of in vitro mutagenesis can be coupled with the ease of yeast genetics to isolate mutants in the human gene. Of particular interest are mutants with a dominant phenotype that can be tested for their effects after introduction back into human cells. For example, conditional dominant lethals could be isolated which prevent function of the wild-type gene by competing out positive effectors.
or substrates. After isolation in yeast these could be used for knocking out gene function in mammalian cells providing an alternative procedure to present techniques such as antibody injection or anti-sense message.

Cell cycle implications

Because the human CDC2Hs can provide all of the functions of cdc2Sp in fission yeast it is reasonable to assume that it performs a similar role to cdc2Sp in controlling the human cell cycle. This conclusion is supported by the structural similarity between the two genes both in overall homology and size of the proteins. But it will be important to establish that CDC2Hs does have this role in human cells.

The most likely points of action of CDC2Hs during the human cell are cycle, analogous to start and the mitotic control in S. pombe, in late G1 at the R-point and in late G2 at the initiation of mitosis (Fig. 6). At the R-point, cells undergo a transition which leads to the cell becoming less dependent on the presence of growth factors and the maintenance of high rates of protein synthesis for completion of the cell cycle. This commitment to the mitotic cell cycle could correspond to the passage of start in the yeasts. Initiation of mitosis has been shown to involve maturation promotion factor (MPF) in vertebrate eggs, and similar activities have been recovered from mammalian mitotic cells and the budding yeast. The activation of MPF is quite analogous to the mitotic control which operates in S. pombe.

Given the homology of CDC2Hs to protein kinases in general and to the cdc2Sp protein kinase specifically it is likely that passage through the two control points in human cell cycle will involve protein phosphorylation. The identification of a

Fig. 6 Comparison of cell cycle control in fission yeast and vertebrate cells. The S. pombe start commitment control is analogous to the vertebrate R-point control, and the S. pombe mitotic control to the G2/M transition regulated by MPF in vertebrate cells.