Isolation and analysis of the fission yeast gene encoding polymerase δ accessory protein PCNA

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Five monoclonal antibodies raised against rat PCNA cross-reacted with a similar protein in the fission yeast *Schizosaccharomyces pombe*. One of these was used to screen an *S.pombe* cDNA expression library. An incomplete cDNA was isolated and used to screen a genomic library, identifying a single gene, designated *pcn1*⁺ (proliferating cell nuclear antigen). The gene encodes a protein of 260 amino acids, with a deduced sequence 52% identical to human and rat PCNAs, which are 98.5% identical to each other. The budding yeast PCNA homologue *POL30* is only 35% identical to the human and rat proteins. *Pcn1* has a region near the C-terminus of particularly high homology to higher eukaryotic PCNA proteins. *pcn1*⁺ is essential for viability and $\Delta pcn1$ cells undergo aberrant DNA replication before cell cycle arrest. Overproduction of the protein leads to cell cycle delay in G₂. Disruption of *pcn1*⁺ is complemented by the human PCNA gene, demonstrating that these genes are functional homologues.

Key words: cell cycle/DNA replication/PCNA/S phase/*Schizosaccharomyces pombe*

Introduction

DNA replication in eukaryotic cells is a complex process requiring the interplay of a number of proteins. Much of our understanding of DNA replication comes from studies of the simian virus 40 (SV40), whose DNA is replicated by cellular proteins together with the viral T antigen (reviewed by Challberg and Kelly, 1989; Stillman, 1989; Fanning and Knippers, 1992). Reconstitution experiments with the SV40 system indicate that lagging strand synthesis requires DNA polymerase α and replication protein A (RPA, RF-A, SSB, HSSB). DNA polymerase δ , replication factor C (RF-C) and proliferating cell nuclear antigen (PCNA) are required in addition to these proteins for leading strand synthesis (Weinberg *et al.*, 1990). If polymerase δ , PCNA or RFC are left out of the reaction, only short lagging strand products are formed (Tsurimoto *et al.*, 1990). Both PCNA and RFC are required for the processivity of polymerase δ (Tsurimoto *et al.*, 1990; Tsurimoto and Stillman, 1991a).

PCNA is also essential for chromosomal DNA replication in higher eukaryotes. Microinjection of anti-PCNA

antibodies into frog oocytes inhibits replication (Zuber *et al.*, 1989), whilst anti-sense oligonucleotides to PCNA inhibit DNA synthesis and cell proliferation in murine Balb/c 3T3 cells (Jaskulski *et al.*, 1988). It is a highly conserved protein found in all eukaryotes examined. Disruption of *POL30*, the *Saccharomyces cerevisiae* homologue, shows that it is an essential gene (Bauer and Burgers, 1990). *Drosophila* PCNA is ~70% identical to the human protein (Yamaguchi *et al.*, 1990), whilst *POL30* is 35% identical. Although *Drosophila* PCNA can substitute for the human protein during *in vitro* SV40 replication, the *S.cerevisiae* protein cannot (Bauer and Burgers, 1988; Ng *et al.*, 1990). *POL30* is able to enhance the processivity of mammalian polymerase δ *in vitro* however, suggesting that its properties overlap with those of higher eukaryotic PCNA (Bauer and Burgers, 1988).

Five of 11 monoclonal antibodies raised against rat PCNA were found to cross-react with a fission yeast protein of similar molecular weight, but not with *POL30* (Waseem and Lane, 1990). This suggested that the *S.pombe* protein may show closer similarity to higher eukaryotic PCNA. In this case, fission yeast would provide a good model system in which to study this protein genetically. The identification of further components of the DNA replication machinery in *S.pombe* would also facilitate studies of the control of chromosomal replication within the cell cycle. To this end, we report here the isolation of *pcn1*⁺, the gene encoding PCNA in *S.pombe*. *Pcn1* is 52% identical to its human equivalent, a considerably higher identity than that reported for the various *S.cerevisiae* homologues of higher eukaryotic DNA replication proteins. We show that *pcn1*⁺ is an essential gene with a role in chromosomal DNA replication and that disruption of *pcn1*⁺ is complemented by the human PCNA gene, demonstrating that the genes are true functional homologues.

Results

Isolation of a gene encoding an *S.pombe* homologue of PCNA

Five of 11 monoclonal antibodies raised against rat PCNA reacted with a protein of similar molecular weight in *S.pombe* (Waseem and Lane, 1990). PC10 showed the strongest reactivity and was used to screen an *S.pombe* λ gt11 cDNA library (Young and Davis, 1983). Three PC10 positive plaques were purified from 10⁵ plaques, one of which showed a positive reaction with the four other anti-PCNA monoclonal antibodies. The insert from this bacteriophage was sequenced, identifying an ORF that had strong homology to the 3' end of human and rat PCNA. A cosmid library containing *S.pombe* genomic DNA was screened with this cDNA and 18 positive clones selected. A 2.1 kb *EcoRI* fragment from one of these was subcloned and sequenced. The complete sequence is shown in Figure 1 and indicates that the original cDNA lacked 84 amino acids at the N-terminus of PCNA.

Characterization of PCNA gene

The gene thus identified was designated *pcn1*⁺ (proliferating cell nuclear antigen). The sequence predicts a 260 amino acid protein with a molecular weight of 28 kDa. Pcn1 shares 52% amino acid identity with human and rat PCNA (Almendral *et al.*, 1987; Matsumoto *et al.*, 1987). Comparison with cDNA sequences of PCNA from other species suggested the presence of a single intron at position 173 in the genomic sequence. A complete cDNA was generated by reverse transcription, amplified by PCR, and sequenced. Examination of this cDNA sequence led to the identification of the intron splice junctions, which are in good agreement with the consensus splice junctions for *S.pombe* (Hoffermann *et al.*, 1990). The intron is 108 bp long and contains three translational termination codons in the predicted open reading frame of *pcn1*⁺.

The transcriptional start site was mapped by primer extension from total RNA. There are two sequences identical to the TATA box at positions -114 and -85. The TATA box is more commonly located 25–30 bp upstream of the start site in *S.pombe* (Russell, 1983). The first initiation codon ATG is at position +93. Various motifs for DNA binding proteins are found in the 5' untranslated region. These include an octamer motif ATGCAAAT (Parslow *et al.*, 1984) at position -240, an upstream control element ACGGAC (Schnapp *et al.*, 1990; Srivastava *et al.*, 1990) at -167 and a CCAATT box at -577. In addition, an AP1 site (Curran and Franza, Jr, 1988) is present at -378. The sequence AAAATAA is repeated four times in the 5' untranslated region, at positions -133, -142, -524 and -571. Comparison with the cDNA shows the polyadenylation site to be at position 1163.

Expression of the complete cDNA in bacteria gave rise to a protein with an apparent molecular weight of 36 kDa which was recognised by monoclonal antibody PC10 on a western blot. A protein of identical molecular weight was detected in extracts of *S.pombe* cells. Western blot analysis of cells synchronised by elutriation (kindly provided by Dr J.Hayles) showed no major variation in the level of Pcn1 during the cell cycle (Figure 2).

Δpcn1 cells show *pcn1*⁺ is essential with a role in DNA replication

A wild type diploid strain auxotrophic for uracil was transformed with a linear fragment containing *pcn1*⁺, in which 330 bp after the intron (43% of the gene) were replaced by *ura4*⁺. Two stable *ura4*⁺ isolates were picked as candidate heterozygous disruptants. A Southern analysis of these two strains is shown in Figure 3. The pattern for one of these, pd17, is consistent with it being heterozygous for the predicted *pcn1* disruption (see legend to Figure 3). The pattern for the other, pd10, is not, and this strain was not pursued further. Tetrad analysis of pd17 was performed. In 22 tetrads, two of the four spores gave rise to haploid colonies that were uracil auxotrophs. Figure 4A shows that the other two spores completed between zero and two cell divisions before arresting with an elongated *cdc*⁻ phenotype, typically between three and five times wild type size. These results demonstrate that *pcn1*⁺ is an essential gene, disruption of which leads to cell cycle arrest.

In order to characterize the nature of the arrest, germination of *Δpcn1* spores was followed in liquid culture. A suspension of spores from a sporulated pd17 culture was

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GAATTCCTGTTAACTGTAATCTACGAATATTGAGAAGAAAAAAGCAATGAATT -694
TGTGTGAATACATATTTTTCCTTAGCGAACAGTACTACTCTTCTATTTCATACCTCTTAGTCTT -623
ATTTAAACAGCAATAAAATTCGTTTAAAGTTTTCGCGTGAATACCAATTAATAATACGTTTTTCAAT -552
CATTTTATATGTCATTGCAACGTGAAAAAAGTTAATGGAATTCGCTAGCAGCTAATATGGAATATGA -481
CAATTTTACTTGATACATTAGTGTAGCTAAATTTTATTGATCTGGAAAGTATATTAAGGTAGAACGCGA -410
AAGCTCTCTAGTTTACGCGGTAATTTGTAATGACTGCTGATTACATTTGGCTATTATATCCGTATAG -339
CTAGAAAAATTCGATAACTGAATATTGCTCTTTAAAAAGACATAAATAGAACCGTGAATTAGTACAGCAA -268
ACGAAAGATAAAAAATTCATAGTAACATGCAAACTACTATTTTTCAGTTAGTTTCCATATTTTGTATAA -196
TATTTTCATATCAACGCGATCTAAAAAGTACGGACGAAATTAATGCTATTTGTAATAAATAAATAACA -125
AAACAAATATAATAACGTAACGCTGATTTTGGTCTCATATATGAAGGTTCTTGAGATTTTACGTTG -54
TCAGCTAGCAATTTTACGACACTATTCTGCTTACGCGCTCTTCTGACTACTTCCCAAGCCAACTACAA 18
AAGGTTTTGTCAGTAACCTTTGTTAGCTATTCTTTATATCTCTATGACTGTATATAAATAAATAAATACG 89
ACT ATG CTT GAA GCT AGA TTT CAG CAG GCT GCT TTG TTG AAA AAG CTT CTC GAT 143
M L E A R F Q Q A A L L K K L L D
GCC ATC AAG GAG TTA GTT ACA GAT GCT AAT GTATGAAGGGGCAGCATATAAATAAGATG 204
A I K E L V T D A N
CTCAACGACAAAGCCTTTCTTTTTTAAAAAATATATTATTAATCTCTTTGAATGATTCTCTAACC 275
TCATAACAG TTT GAT TGC AAT GAC AAC GGT ATC TCT CTG CAG GCT ATG GAT AGT 329
F D C N D N G I S L Q A M D S
TCT CAT GTA GCC CTT GTT TCT ATG CTT ATT AAA TCA GAT GGA TTT GAA CCA TAT 383
S H V A L V S M L I K S D G F E P Y
CGG TGT GAT AGA AAT ATT GCT TTA GGT ATA AAT CTG AAC GCC TTA TCA AAG GTC 437
R C D R N I A L G I N L N A L S K V
TTA CGG TGT GCC CAA AAC GAG GAT TTG GTT ACT TTA AAG GCT GAA GAT ACA CCT 491
L R C A Q N E D L V T L K A E D T F
GAG GTA TTG AAT TTG GTT TTC GAG AGC GAG AAA AAT GAC AGG ATC TCT GAT TAT 545
E V L N L V F E S E K N D R I S D Y
GAT GTT AAA TTA ATG GAC ATT GAT CAA GAA CAC TTG GGT ATA CCA GAT ATC GAA 599
D V K L M D I D Q E H L G I P D I E
TAC GAT GCT ACT ATT ACT ATG CCT GCT GCC GAA TTT CAA CGC ATT ACT CGA GAT 653
Y D A T I T M P A A E F Q R I T R D
TTA TTA ACT TTG AGT GAT TCA GTA ACA ATC AAT GCT AGT AAA GAG GGT GTT CGT 707
L L T L S D S V T I N A S K E G V R
TTT AGC TGC AAG GGA GAT ATT GGT AAT GGT AGT ACC ACG CTG AAA CAA CAT ACC 761
F S C K G D I G N G S T T L K Q H T
GAT CTT TCT GAC CAA GAT CAA AGT ATC GAA ATT TCT CTT ACC CAA GCT GTA ACA 815
D L S D Q D Q S I E I S L T Q A V T
CTT ACG TTT TCT TTG AAA TAT TTA GCC CAG TTC ACT AAG GCT ACT CPT TTA GCT 869
L T F S L K Y L A Q F T K A T P L A
ACC CGA GTC ACA CTT TCA ATG AGC AAT GAT GTT CCA CTT CTT GTG GAG TAT AAA 923
T R V T L S M S N D V P L L V E Y K
ATG GAA TCC GGA TTT TTA CGA TTT TAT CTT GCC CCA AAA ATT GGT GAG GAG GAT 977
M E S G F L R F Y L A P K I G E E D
GAG GAG TAG ATTACGGAATCAATATCTTTAGTAATCTCTTTGCAATTGACGAGCTAGCATCATTATA 1045
E E
AACATACCTTTGTTTACTTTTAAATCAACCTTTTGTACTGCTCTCCCTCAATTCATATCCAAACA 1116
*polyA tail
GGCAAAATACGAGCTTTTACTTAAAAAATAAATACTTCTTGAACCAACAACTGAAGGGTAGAAAACT 1187
TGTGTTCTTAGTGATGATTTTTCACAGATAACTTCGTAATCATCTGCTGATGCTTGTGATTAAGCTAG 1258
TACATGATATAATACATTGATATGTAAATTAGCTTAATCTGAGAATTTTATTCTAACTGATAATTGTATAC 1329
TTATATGGCTAACCGCGTAAGAGCCTCGGAGGGGACAGTTCGCCAAATACTACCAACGCTACCCACGATAG 1400
CAGCAAGGCGTAAAGTTACTTAAAGAAGATGAAATTC 1438

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Fig. 1. Complete nucleotide sequence and deduced amino acid sequence of *pcn1*⁺. The transcriptional start site is marked by +1 and the poly(A) addition site is marked at the 3' end of the gene. The consensus splice junctions of the intron are underlined. The complete sequence of the *EcoRI* fragment containing the PCNA gene has been entered into EMBL Data Library under accession number X54857.

made according to the method of Moreno *et al.* (1989). In this spore preparation, half of the spores were uracil auxotrophs and *pcn1*⁺, whilst half were prototrophic for uracil and *Δpcn1*. This meant that in medium lacking uracil,

Fig. 2. The level of Pcn1 protein shows no major variation during the cell cycle. Wild type 972h⁻ cells were synchronized by centrifugal elutriation and then cultured in minimal medium at 32°C, samples being taken every 20 min. The peaks in cell plate index are equivalent to the midpoint of S phase (Nasmyth *et al.*, 1979). The level of p36^{pcn1} as monitored by Western blotting is shown between 60 and 220 min. This represents a complete cell cycle.

only the $\Delta pcn1$ spores would germinate. As a control, a similar spore preparation was made in which half the spores were uracil prototrophs and $pcn1^+$, using a wild type diploid strain heterozygous for a $ura4^-$ mutation.

Cell number, nuclear morphology and DNA content were monitored during a time-course of germination. The $\Delta pcn1$ culture showed a total increase in cell number of 1.6 times the original value (Figure 4B). This correlated well with the predicted increase of 1.63, calculated from the tetrad data in Figure 4A, taking into account that only the 50% of the spores that are ura^+ should germinate. In the control culture, cell number began to increase after ~10 h and then increased exponentially.

Nuclear morphology was monitored by staining cells with the DNA binding dye DAPI (Figure 5A). For the wild type control, first signs of germination were seen after 5 h, whilst 50% had germinated by 8 h. By 13 h most cells were dividing normally. The exponential growth and division of the ura^+ cells diluted out the non-germinating ura^- spores. Germination of $\Delta pcn1$ spores proceeded similarly and by 13 h ~80–85% of the germinating ura^+ cells were seen to be elongate with an interphase nucleus. The remainder had abnormal mitotic features which accumulated at later times. An example from the 17 h time-point is shown in the bottom right-hand panel of Figure 5A. Such abnormalities were not observed in the wild type control.

The DNA content of the germinating spores was also followed during the same time-course using flow cytometry (Figure 5B). Initially the spores were in G₁, with a 1C DNA content. In the wild type control cells that had completed DNA replication were first seen at 7 h and comprised 50% of the cells by ~10 h.

For the $\Delta pcn1$ spores DNA replication was also first seen at 7 h. In contrast to the control, however, germinated cells were seen to accumulate in S phase throughout the period during which wild type ura^+ cells complete their first S

phase. At later time-points a broad peak was observed including many cells with DNA contents between 1C and 2C, suggesting that some cells were delayed in progression through S phase, or had arrested with partly replicated DNA.

To investigate this further, an assay for incomplete or imperfect replication was employed that had been previously used in *S.cerevisiae*. Hennessy *et al.* (1991) showed that defectively replicated chromosomes could not be separated by pulse field gel electrophoresis (PFGE), in contrast to fully replicated chromosomes. *S.pombe* contains three chromosomes of 3.5, 4.6 and 5.7 Mb, which can also be separated using PFGE (Smith *et al.*, 1987; Fan *et al.*, 1989). Figure 6 shows the results of an experiment using such a PFGE system (Southern *et al.*, 1987).

As for *S.cerevisiae*, when cells were treated with hydroxyurea the genome did not resolve as three chromosomes (Figure 6, lane 7). When samples were prepared from $\Delta pcn1$ cells at times equivalent either to the peak of the abnormal first S phase or to the terminal state, the three chromosomes similarly failed to resolve, in comparison with the appropriate controls (Figure 6, lanes 2–5). These results suggest that after germinating and entering an abnormal first S phase, $\Delta pcn1$ cells arrest with DNA that has not completed replication in the normal fashion.

Overproduction of *pcn1*

To investigate the effect of overproducing $pcn1^+$, the ORF was cloned into pREP1 (kindly provided by Dr K. Maundrell). This vector contains the *nmt1*⁺ promoter (Maundrell, 1990), which is repressed by thiamine and induced in its absence, induction peaking after 16 h at 32°C. CAT assays have shown that the induced level is ~80 times higher than the repressed level and ~6 times greater than that produced by the highly active *adh1* promoter (Basi *et al.*, 1992).

Fig. 3. Southern blot analysis of *pcn1* disruption. (A) Predicted structure of *pcn1*⁺ and Δ *pcn1* genomic regions. In Δ *pcn1* the *Pst*I–*Xho*I fragment of *pcn1*⁺ is replaced by *ura4*⁺ (the hatched region represents the *ura4*⁺ gene, the stippled region representing small regions of plasmid DNA present in the fragment). (B) Southern blot of chromosomal DNA from the parental diploid strain (lanes 1, 4 and 7) and two putative heterozygous disruptants (pd17, lanes 2, 5 and 8; pd10, lanes 3, 6 and 9). DNA was digested with *Eco*RI (lanes 1–3), *Eco*RV (lanes 4–6) or *Hind*III (lanes 7–9) and probed with the *pcn1*⁺ *Eco*RI fragment. The parental strain shows a single band with *Eco*RI (since the blot is probed with this fragment) and two bands with *Eco*RV and *Hind*III (since *pcn1*⁺ contains a single site for each of these enzymes and so the gene is split between two fragments, which also contain flanking sequences). pd17 shows all of the parental bands, together with other bands (two *Eco*RI, two *Eco*RV and one *Hind*III), whose sizes correspond precisely with the predicted disruption. pd10 also shares the parental bands, but has other bands that do not correspond to the disruption. (C) The same blot was probed with *ura4*⁺. No bands are seen with the parental diploid strain, which is deleted for *ura4*⁺. The pattern for pd17 again corresponds to the predicted disruption (one band of ~2.6 kb with *Eco*RI, two bands with *Eco*RV and one band of ~1.8 kb with *Hind*III). pd10 clearly possesses the *ura4* gene, but this appears to be at some site other than the *pcn1* locus.

Fig. 4. $\Delta pcn1$ spores arrest with a *cdc*⁻ phenotype after 0–2 divisions. (A) Summary of the terminal phenotypes of 44 $\Delta pcn1$ spores from 22 tetrads. After completing 0–2 divisions the cells arrested and elongated, typically reaching three to five times the size of a wild type cell. (B) Increase in cell number during the germination of wild type or $\Delta pcn1$ spores. Experiments were performed at 32°C in minimal medium lacking uracil, so that only the 50% of spores that were *ura*⁺ should have germinated (see text).

Induction of *pcn1*⁺ increased the generation time 2- to 3-fold (Figure 7A). Most cells became elongated with a single interphase nucleus (Figure 7B), although after 20 h a low percentage of cells (~14%) showed a 'cut' phenotype, in which a septum cuts through the nucleus (examples are shown in Figure 7B). This phenotype is suggestive of the failure of chromosomes to segregate normally during mitosis (Uemura and Yanagida, 1984) and is reminiscent of the infrequent mitotic abnormalities seen with $\Delta pcn1$ cells (Figure 5A).

Flow cytometry showed a 2C peak with a smear to higher levels (Figure 7C). The smear is likely to be due to 'cut' cells in which chromosome segregation at mitosis is abnormal, resulting in greater than 2C DNA contents after the following S phase. The possibility that the 2C peak might still represent incompletely replicated DNA, as with the

Fig. 5. $\Delta pcn1$ spores arrest after undergoing abnormal DNA synthesis. (A) Examples of germinating $\Delta pcn1$ and wild type spores stained with the DNA binding dye DAPI, after 8, 13 and 17 h at 32°C. For $\Delta pcn1$ at 17 h, an example is also shown of a cell in which the nucleus is cut through by a septum (right-hand panel). (B) DNA content of $\Delta pcn1$ and wild type spores was measured by flow cytometry after 4, 5, 7, 8, 9, 13 and 17 h of germination at 32°C. The $\Delta pcn1$ 13 and 17 h time-points are gated to exclude spores that have not germinated. The positions of 1C and 2C controls are marked.

terminal $\Delta pcn1$ phenotype, was investigated by PFGE (Figure 6, lanes 8 and 9). Chromosomes from cells in which *pcn1*⁺ had been induced for 20 h entered the gel normally,

Fig. 6. Defectively replicated chromosomes cannot be separated by pulse field gel electrophoresis. Samples were run for 7 days using a voltage gradient of 1 V/cm and a pulse interval of 1 h (see Materials and methods). Chromosome preparations were from the following cells: 972h⁻ (lanes 1 and 10); $\Delta pcn1$ spores after 9 and 13 h germination (lanes 2 and 3); wild type spores after 9 and 13 h (lanes 4 and 5); *leu1-32 h⁻* cells growing exponentially (lane 6) or treated with 10 mM HU for 3 h (lane 7); *leu1-32 h⁻* transformed with pREP1*pcn1* (lane 8) or pREP1 (lane 9) and induced for 20 h at 32°C. The loading across the gel varied by no more than 2- to 3-fold.

suggesting that overproduction of *pcn1*⁺ probably allows completion of DNA replication, although it results in cells delaying in G₂.

The effect of overproducing *pcn1*⁺ in a *cdc2-3w* strain was examined. *cdc2-3w* is a semi-dominant, gain-of-function mutation of the fission yeast *cdc2*⁺ gene, which causes cells to divide at a reduced cell size (Fantès, 1981). When DNA replication is inhibited by hydroxyurea, *cdc2-3w* cells still enter mitosis (Enoch and Nurse, 1990). This suggests that the mutant is defective in mitotic checkpoint control (for a discussion of such 'checkpoints' see Hartwell and Weinert, 1989; Enoch and Nurse, 1991). It was subsequently shown that when *cdc2-3w* is crossed with a variety of mutants that cause cell cycle arrest in either S phase or G₂, the double mutants still enter mitosis at the restrictive temperature, generating a 'cut' phenotype (Enoch *et al.*, 1991). This suggests that the checkpoint which is defective in *cdc2-3w* links mitosis to a large number of interphase events. It was thus of interest to determine whether *pcn1*⁺ overproduction delayed mitosis by means of this checkpoint.

When *pcn1* was induced in *cdc2-3w*, signs of a cell cycle delay were first seen after 12 h. By 16 h, only 2% of cells showed a 'cut' phenotype; 64% of cells were elongated with an interphase nucleus, whilst the remaining 34% represented cells that had lost the plasmid. Flow cytometry indicated that cells were elongating with a 2C DNA content and were therefore in G₂ (data not shown). These results show that the G₂ delay produced by *pcn1* overproduction is not mediated by the checkpoint which is defective in *cdc2-3w*. This suggests that another mechanism must exist that blocks onset of mitosis when cell cycle progression is delayed by overproduction of *pcn1*⁺.

Δpcn1 is complemented by the human PCNA gene

The isolation of yeast genes with sequence homology to higher eukaryotic genes does not demonstrate that these genes are functional homologues. In order to investigate whether *pcn1*⁺ really is functionally equivalent to mammalian PCNA, the ability of the human PCNA gene to complement the *pcn1* disruption was investigated.

cDNA encoding human PCNA and *POL30*, the *S.cerevisiae* PCNA gene (kindly provided by Dr B.Stillman) were cloned into pREP1. The plasmids were then transformed into pd17 (the diploid strain heterozygous for

the *pcn1* disruption) as were REP1*pcn1*, REP1 and pIRT2-*pcn1*. pIRT2*pcn1* is a multicopy vector carrying the 2.1 kb genomic fragment containing *pcn1*⁺ driven by its own promoter. Cells were sporulated and the spores spread on plates lacking uracil, so that only $\Delta pcn1$ spores could germinate. For the REP1 constructs the experiment was performed with the promoter either induced or repressed.

pIRT2*pcn1* complemented the disruption fully. Human PCNA was also seen to complement, but only with the promoter induced. $\Delta pcn1$ cells containing the human PCNA cDNA divided with a generation time of 6.7 h at 32°C, compared with 4.0 h for a wild type strain expressing human PCNA to the same level. DAPI stained $\Delta pcn1$ cells carrying the human PCNA cDNA were wild type in appearance and were shown by flow cytometry to be haploid (data not shown).

Neither *pcn1*⁺ nor *POL30* rescued $\Delta pcn1$ cells when the cDNAs were expressed from the *nmt1* promoter in these experiments. The reason for this (particularly the former) remains unclear. When overproduced in either haploid or diploid cells, both of these genes perturb the cell cycle and increase the generation time more severely than the human gene (see above; data not shown for *POL30*). Thus failure to rescue may have been due to this effect when the promoter was induced and to insufficient expression when the promoter was repressed. The effects of *pcn1*⁺ overproduction may be more pronounced in germinating spores. In addition there may be a requirement for regulated expression of *pcn1*⁺.

Discussion

We have used monoclonal antibodies raised against rat PCNA to isolate its homologue in fission yeast. The cDNA isolated was then used to clone the corresponding gene, including 843 bp 5' and 452 bp 3' of the ORF. A complete cDNA was generated and expressed in *Escherichia coli*, yielding a protein which was recognized by PC10, the monoclonal antibody used to clone the cDNA.

A comparison of the amino acid sequence of *pcn1*⁺ with the sequences of the human and *S.cerevisiae* PCNA proteins is shown in Figure 8. The helix-turn-helix motif, a putative DNA binding domain, is conserved in *pcn1*⁺ between residues 61 and 77, with a few preferred amino acid substitutions. Rat PCNA differs from the human protein by four amino acids and the two proteins are 98.5% identical. Pcn1 shares 52% overall amino acid identity with human and rat PCNA and 70% over the C-terminal 56 amino acids. Whilst *Xenopus* PCNA is 89% identical to human, *Drosophila* 70% and rice PCNA 62%, budding yeast *POL30* shares only 35% identity with the human protein (Bauer and Burgers, 1990; Leibovici *et al.*, 1990; Yamaguchi *et al.*, 1990; Suzuka *et al.*, 1991). The C-terminus of *POL30* is quite divergent from that of the other PCNA proteins. Seventeen of the 25 amino acids between residues 216 and 240 are identical in *S.pombe*, rat, human, *Drosophila* and *Xenopus* PCNA proteins, whereas only four of these residues are present in *S.cerevisiae*. It is possible that the C-terminus plays a key role in determining the specificity of PCNA interaction with other proteins, such as polymerase δ and RF-C. This could explain why *Drosophila* PCNA can replace the human protein during *in vitro* SV40 replication, whereas budding yeast PCNA cannot (Ng *et al.*, 1990). It will be of interest to determine whether *S.pombe* Pcn1 can complement in this fashion.

Fig. 7. Overproduction of *pcn1*⁺ causes cell cycle delay in *G₂. leu1-32 h⁻* was transformed with pREP1*pcn1* or pREP1 and the promoter induced at 32°C as described in Materials and methods. (A) Cell number was measured at various times of induction using a Coulter counter. (B) Examples of cells fixed in ethanol after 0, 12 and 20 h induction and stained with the DNA binding dye DAPI. For REP1*pcn1* at 20 h, the right-hand panel shows a cell in which the nucleus is cut through by a septum (the left-hand panel contains another example together with cells that are elongating with an interphase nucleus). (C) DNA content as measured by flow cytometry is shown at 0, 12, 18 and 20 h of induction. The positions of 1C and 2C controls are marked.

In order to study the function of *pcn1*⁺ in *S.pombe*, a disruption was constructed which resulted in 43% of the gene being deleted. Δ*pcn1* spores went through an abnormal S phase after germinating and completed between zero and two cell divisions, before arresting as elongated cells with DNA that was incompletely or improperly replicated. This phenotype parallels that seen when individual disruptions are made of the genes encoding *S.cerevisiae* homologues of higher eukaryotic polymerases α, δ and ε, as well as RP-A and PCNA (Johnson *et al.*, 1985; Bauer and Burgers, 1988; Heyer *et al.*, 1990; Morrison *et al.*, 1990). However the

terminal DNA content was not determined for any of these gene disruptions.

As stated above, Δ*pcn1* spores arrest with DNA mostly, but not properly replicated. It appears that DNA replication is impaired, but not immediately inhibited, in the absence of PCNA or with reduced levels of PCNA if there is any carry-over of protein in the spores. It is interesting to note that in *S.cerevisiae*, temperature sensitive *cdc2* mutants that are defective in polymerase δ arrest with 70% of their DNA replicated (Conrad and Newlon, 1983). This is in contrast to mutants defective in polymerase α, which arrest with only

<i>S.cerevisiae</i>	MLEAKFEEASLFKRIIDGFKDCVQLVNFQCKEDGIIAQAVDDSRVLLVLSLEIGVEAFQEQYRCDHPVTI
<i>S.pombe</i>	MLEARFQQAALLKKLLDAIKELVTDANFDCNDNGISLQAMDSSSHVALVSMLIKSDGFEPYRCDRNIAL
<i>H.sapiens</i>	MFEARLVQGSILKKVLEAIKDLINACWDISSSGVNLQSMDSHSVSLVQLTLRSEGFDTYRCDRNIALM
<i>S.cerevisiae</i>	GMDLTSLSKILRCGNNTDTLTLLIADNTPDSIIILFEDTKKDRIAESYSLKIMDIDADFLKIEELODYDST
<i>S.pombe</i>	GINLNALSQVLRCAONEDLVTLKAEDTPEVLNLVFESEKNDRI SDYDVKLMDIDOEHLGIPDIEYDAT
<i>H.sapiens</i>	GVNLTSMISKILKCAINEDIIITLRAEDNADTLALVFEAPNQEKVSDYEMKLMIDLVEQLGIPEQEQYSCV
<i>S.cerevisiae</i>	LSPSPSEFSKIVRDLSQLSDSINIMITKETIKFVADGDIGSGSVI IKPFVDMHPETS IKLEMDQPDV
<i>S.pombe</i>	ITMPAAEFQRITRDLLTSLSDSVTINASKEGVRFSCKGDIGNGSTTLKQHTDLSDDQDSIEISLTQAVT
<i>H.sapiens</i>	VKMPSGEFARICRDLSHIGDAVVISCAKDGVKFSASGELGNGNIKLSQTSNVDKEEEAVTIEMNEPVQ
<i>S.cerevisiae</i>	LTFGAKYLLDIIKGSLSLDRVGIRLSSEAPAL-FQFDLKSGLQFFLAPK--FNDEE
<i>S.pombe</i>	LTFSLKYLAQFTKATPLATRVTLMSNDVPLLVVEYK-MESGFLRFYLAPKIGEEDEE
<i>H.sapiens</i>	LTFALRYLNFFTKATPLSSTVTLMSADVPLVVEYKIADMGHLKYLLAPKI--EDEEGS

Fig. 8. Comparison of the predicted amino acid sequence of human PCNA with those of the *S.pombe* and *S.cerevisiae* homologues. Amino acids shown in bold are identical in the human sequence and either or both yeast proteins.

25% replication completed (Lucchini *et al.*, 1990). Chromosomal DNA replication seems to have a more stringent requirement for polymerase α than for δ or its cofactor PCNA, although all these proteins are essential for viability.

These results can be accounted for by recent data from studies of SV40 replication *in vitro*. Polymerase α has been shown to be responsible for primer formation and lagging strand synthesis, whilst δ acts in association with PCNA and RF-C, to effect leading strand synthesis (reviewed by Challberg and Kelly, 1989; Bambara and Jesse, 1991; Wang, 1991). PCNA only binds to DNA in association with RF-C or polymerase δ (Tsurimoto and Stillman, 1991b). The RF-C-PCNA complex binds to the primer junction and has the dual role of facilitating the commencement of synthesis by δ , as well as preventing α from binding (Tsurimoto and Stillman, 1991a). This provides a model for the initiation of leading strand synthesis and also suggests that in the absence of PCNA, polymerase α might be able to extend the primers that it has formed and thus contribute to leading strand synthesis. Such abnormal synthesis by polymerase α has in fact been observed in an SV40-based system lacking PCNA and RF-C (Ishimi *et al.*, 1988) and could provide part of the explanation for the $\Delta pcn1$ phenotype. A gene encoding an *S.pombe* homologue of the polymerase α catalytic subunit has recently been described (Damagnez *et al.*, 1991).

There is also evidence that both RF-C and PCNA are required for the processivity of polymerase δ , once leading strand synthesis has begun (Tsurimoto and Stillman, 1991a). It is worth noting that the role of polymerase ϵ has yet to be well defined in the cell and that this could also provide an explanation for residual synthesis in the absence of PCNA.

It is interesting that overproduction of *pcn1*⁺ appears to cause cell cycle delay in G₂. The effect is seen even in *cdc2-3w*, a strain defective in a checkpoint linking mitosis to the completion of S phase (Enoch and Nurse, 1990). This suggests that the delay is not mediated by this checkpoint. It is possible that *pcn1*⁺ induction mimics some other signal, to which *cdc2-3w* still remains responsive. For example, *cdc2-3w* does not differ significantly from wild type cells in its sensitivity to UV (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992). This suggests that the checkpoint

which causes a G₂ delay in response to DNA damage (Hartwell and Weinert, 1988; Al-Khodairy and Carr, 1992) may still be intact, although this remains to be demonstrated directly. PCNA has been implicated in DNA repair in response to UV irradiation (Celis and Madsen, 1986; Toschi and Bravo, 1988; Shivji *et al.*, 1992).

The ability of the Human PCNA cDNA to complement a disruption of *pcn1*⁺ suggests close similarities between the replication machineries of fission yeast and mammalian cells. Although studies of DNA replication are considerably more advanced in *S.cerevisiae* than in *S.pombe*, the high degree of homology between *pcn1*⁺ and its human equivalent suggests that further studies in the fission yeast will be of value. The identification of more elements of the replication machinery in *S.pombe* will also aid our understanding of how DNA replication is controlled within the eukaryotic cell cycle.

Materials and methods

S.pombe methods and strains

Unless mentioned below, all genetical and biochemical procedures involving *S.pombe* were as described by Moreno *et al.* (1990). Cells were fixed, stained and analysed for flow cytometry according to the method of Sazer and Sherwood (1990). A 1C control was prepared using 972h⁻ cells starved for nitrogen, whilst exponentially growing cells were used as a 2C control. A synchronous culture of 972h⁻ cells was prepared as described by Aves *et al.* (1985). The following diploid strain was used for gene disruption: *ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 h⁺/h⁻*. The diploid strain used to prepare the control preparations for spore germination experiments was *ade6-704/ade6⁺ ura4-27/ura4⁺ leu1-32/leu1⁺ h⁺/h⁻*. For overproduction of PCNA the following strains were used: *leu1-32 h⁻* and *cdc2-3w ade6-M210 leu1-32 h⁻*.

Screening of genomic library

Filters containing an *S.pombe* genomic library in a cosmid vector were kindly provided by Dr G.Zehetner (Imperial Cancer Research Fund, London). The *EcoRI* fragment of the *pcn1* partial cDNA was labelled by random hexamer priming (Maniatis *et al.*, 1982) and used to screen the genomic library. Positive colonies were selected and further analysed by Southern blotting. About 1 µg of cosmid DNA from each of the clones to be tested was digested with *EcoRI* or *EcoRV* and analysed on a 0.8% agarose gel. The DNA was transferred to Hybond N⁺ membrane for 4 h and fixed by UV cross-linking. A 581 bp *EcoRI*-*NheI* fragment of the *pcn1* partial cDNA was labelled by hexamer priming and used to probe the Southern blots. Hybridization and washes were carried out under high stringency conditions. The cDNA probe hybridized with a single band of ~2.1 kb within the *EcoRI* digest and bands of 5.5 and 1.8 kb were obtained with the *EcoRV* digest. Three clones gave patterns identical to the genomic digest, suggesting that

they had not undergone rearrangement. A 2.1 kb *EcoRI* fragment from one of these clones (ICRF60D1016), was subcloned into pUR292 and sequenced from both ends. Sequencing reactions were carried out using a 'Sequenase' sequencing kit (United States Biochemicals) and 21mer synthetic oligonucleotide primers.

Preparation of RNA

Total RNA from *S.pombe* was prepared, essentially according to the method of Maniatis *et al.* (1982). Cells from a mid-log phase culture were digested with Novozyme 234 (Novo Biolabs, Denmark) in order to remove their cell walls. The cells were centrifuged and the pellet dissolved in 4 M guanidine thiocyanate containing 1 mM EDTA. The solution was centrifuged again and the supernatant loaded on a cushion of 5.7 M cesium chloride and centrifuged in a SW41 rotor at 126 000 g for 20 h. The pellet was washed with 70% ethanol and suspended in 10 mM Tris pH 7.5, 1 mM EDTA, containing 0.2% SDS, and then ethanol precipitated. After washing the pellet with 70% ethanol, it was dissolved in DEPC treated water.

Synthesis and cloning of cDNA

The cDNA was synthesized using antisense primer 1: 5'-CTGTTTGG-ATATGAATG-3' corresponding to position 1117 in the *pcn1* genomic fragment. For cDNA synthesis, ~10 µg of RNA were taken and annealed with primer 1 in 10 mM PIPES pH 6.4, 400 mM KCl and 2 units/µl of RNasin. The reverse transcription reaction was carried out at 42°C for 2 h in 222.2 mM Tris-HCl pH 8.3, 4.4 mM DTT, 6.7 mM MgCl₂, 1.1 mM of each dNTP, 0.11 units/µl of RNasin and 20 units of avian myeloblastosis virus reverse transcriptase (Promega, USA).

The cDNA obtained after the reverse transcription reaction was amplified using the PCR. This reaction utilised primer 2: 5'-TACGCATATGCTT-GAAGCTAG-3' and primer 3: 5'-TTGATTCGGATCCCTACTCCTCA-TCCT-3'. Primer 2 has an *NdeI* site just upstream of the translational start site and Primer 3 has a *BamHI* site just after the translational-termination codon. After amplification with these primers, the product was digested with *NdeI* and *BamHI* and cloned into *NdeI* and *BamHI* site of pT7.7 (Tabor and Richardson, 1985). The cDNA was sequenced as described above.

Primer extension

Primer 4: 5'-ATCTGTAACCTAAGCTCTTGAT-3', corresponding to position 167 of the *pcn1* genomic fragment, was used for the primer extension reaction. This primer was labelled with ³²P at its 5' end according to the method of Maniatis *et al.* (1982). A reverse transcription reaction on total RNA was carried out as described above. The product was treated with phenol-chloroform, ethanol precipitated and taken up in Sequenase stop solution. The product was analysed on a 6% denaturing gel. Adjacent lanes contained the products of a sequencing reaction of the genomic DNA performed with the same primer.

Construction of plasmid pURδ1016.ura4 and its use in *pcn1* gene disruption

A 1.8 kb *HindIII* fragment from pIRT2U containing the *ura4*⁺ gene was cloned into *HindIII*-digested pBluescript. *ura4*⁺ was then removed from this construct as a *PstI*-*XhoI* fragment and cloned into *PstI* and *XhoI* digested pUR1016 to give pURδ1016.ura4. This resulted in the removal of 330 bp from position 311 in *pcn1*⁺ (see Figure 1).

pURδ1016.ura4 was then digested with *NdeI* and *XmnI*. A 3.4 kb fragment containing *pcn1* disrupted by *ura4* was gel purified and transformed into the diploid strain described above. Transformants were tested for stability of the *ura*⁺ phenotype, by streaking on non-selective medium and then replica plating back to plates lacking uracil.

Pulse field gel electrophoresis

The apparatus used was that described by Southern *et al.* (1987). Plugs were prepared from cells grown in minimal medium as follows. Cells were washed twice in CSE (20 mM citrate-phosphate pH 5.6, 40 mM EDTA and 1.2 M sorbitol) and then resuspended in 10 ml CSE with 15 mg Zymolyase-20T (Seikagaku Kogyo Co. Ltd, Tokyo). After digestion for 1 h at 37°C, cells were pelleted and resuspended at 6 × 10⁸ cells/ml in TSE (10 mM Tris-HCl pH 7.5, 45 mM EDTA and 0.9 M sorbitol). After warming to 37°C, an equal volume of 1% LGT agarose in TSE was added and 100 µl aliquots dispensed to a plug mould. Cells were lysed by incubating plugs in 0.25 M EDTA, 50 mM Tris-HCl pH 7.5 and 1% SDS for 90 min at 55°C. The plugs were then transferred to 1% lauryl sarcosine and 0.5 M EDTA pH 9.5, with 0.5 mg/ml proteinase K and digestion continued at 55°C for 48 h. Plugs were stored at 4°C and equilibrated in TE before loading.

Gels were run at a voltage gradient of 1 V/cm for 7 days, the buffer being changed on the fourth day. Ethidium bromide was used to stain the gels after running.

Overexpression of *pcn1*⁺

Cells transformed with pREP1 or pREP1*pcn1*, were grown in minimal medium with 5 µg/ml thiamine. In order to induce expression from the *nut1* promoter, cells were washed three times in water and then resuspended in minimal medium lacking thiamine.

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